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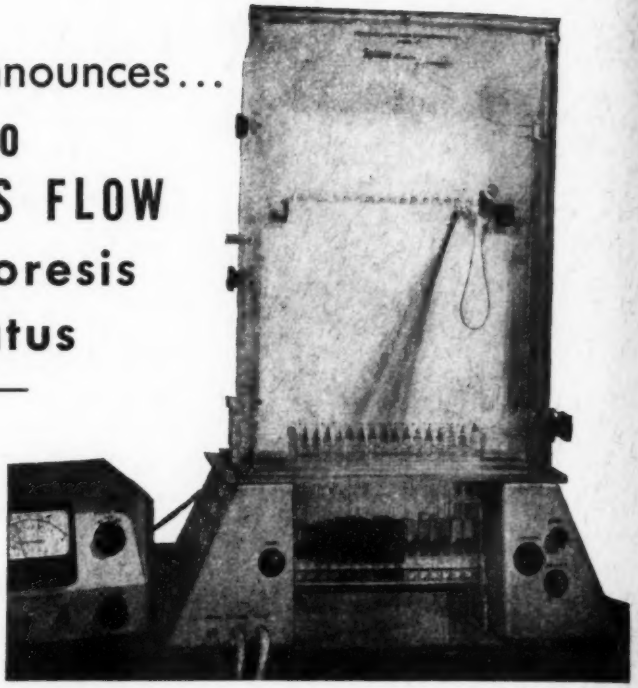

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THE
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A BIMODAL RESPONSE TO DIETARY SUGARS BY AN INSECT[1]

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The gustatory responses of different animal species to sugars have been studied by many workers, and the apparent universality of animal acceptance of sucrose has been pointed out by Frings (1946). The biological importance of carbohydrates and their lack of olfactory-stimulating properties makes them well suited for experimental studies of gustatory responses. As discussed by Roeder (1952), insects are excellent experimental animals for studies in several areas of sensory physiology, owing to their small size, ease of culturing in large numbers, and relative simplicity of behavior. Several different aspects of the gustatory reactions of insects to sugars have been investigated: taste-acceptance thresholds have been determined; the influence of sugars on feeding behavior has been studied; and the relationships between nutritive value and taste-acceptability have been investigated (for review see Dethier, 1953).

As part of a study of the behavior of the larva of the European corn borer, *Pyrausta nubilalis* (Hübn.), on its principal host plant, an investigation was undertaken of the influence of dietary sugars on larval feeding behavior under controlled experimental conditions. The larvae were found to be very sensitive to sugars (Beck, 1956a), and the concentration of sugars in the tissues of the corn plant was shown to influence the orientation of the insect on the plant (Beck, 1956b). The experimental method developed during the course of this research made possible the quantitative measurement of the influence of gustatory substances on feeding behavior. The nature of the measured feeding response and a discovered bimodality of the insect's response to low concentrations of sugar constitute the subject matter of the present report.

Methods

The experimental method employed was of a multiple choice design, in which newly hatched, unfed European corn borer larvae were allowed to choose the dietary media on which to feed. The agar-based medium shown in Table I was used as the nutritional substrate. Agar-gel alone was of a consistency unacceptable to the insects; the addition of casein and cellulose resulted in a medium on which

[1] Approved for publication by the director of the Wisconsin Agricultural Experiment Station. This research was supported in part by the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation.

the larvae would feed. The amounts of sugars employed in these experiments were far too minute to necessitate the adjustment of the amounts of the other constituents to compensate for the slight changes in volume and concentration.

The media were mixed thoroughly with a motor-driven stirrer while being held in a boiling water bath, and then poured into Petri plates (15 mm. × 90 mm.) to gel. From the gelled media, round discs were cut by means of glass tube 18 mm. in diameter. The discs were transferred to Petri plates where they were placed at equal distances apart in a circle, the center of which was the center of the dish. The discs of diet so placed are referred to hereafter as feeding stations. In two-choice experiments there were four feeding stations per Petri dish, each medium being represented twice. The similar media were placed opposite each other in the four-membered circle. In four-choice experiments, eight feeding stations were present, each medium being represented at two stations, and the relative positions of the different media were randomized according to a table of random numbers. The position-randomization of the media in four-choice experiments was modified only to the extent that two identical diets were never placed adjacent to each other. This modification of the random distribution was found to be necessary in order to avoid position effect errors. About 40 European corn borer eggs in the black-head

TABLE I

Composition of agar-based media used to test the feeding reactions of newly-hatched larvae of the European corn borer

Substance	Amount (g)
Bacto-agar	1.10
Fibrous cellulose	0.50
Powdered cellulose	2.00
Casein, vitamin free	3.00
Glucose (or other sugar)	variable
Distilled water	40.00 (ml.)

stage (and about four hours from hatching) were placed on a small piece of moist blotting paper at the center of dish. The lids of the Petri dishes were lined with tightly fitting discs of blotting paper to aid in preventing the larvae from escaping. Eight replicate Petri dishes were set up in each experiment. After the experiment was set up, the dishes were placed in the dark at 30° C. for 24 hours. At the end of this time, the dishes were examined and the number of larvae established at each feeding station was recorded. No counts were made of larvae not on feeding stations. The counts of the number of larvae established at each feeding station were interpreted as reflecting the influence of the experimental diets on the maintenance of larval feeding over the experimental period. Preliminary experimentation demonstrated that the distribution of larvae among the feeding stations was random when the feeding stations were of identical diets.

The physical characteristics of the various media were found to be quite uniform. The larvae were found to be relatively insensitive to small differences in pH and water content; small variations in these properties did not represent a source of experimental error. It was found unnecessary to maintain aseptic conditions in the Petri dishes when the incubation period was 24 hours. With a 48-hour incubation period, the use of aseptic techniques was necessary. However, the larval

establishment data at 24 hours were indistinguishable from those at 48 hours, so the shorter time and simpler technique were adopted.

The experimental data were treated statistically by analysis of variance, after being subjected to square root transformation as recommended by Snedecor (1946) for treatment of small numbers. Regressions were calculated by the method of least squares.

Results and Discussion

The nature of response to sugar. Four-choice experiments, in which the larvae were offered dietary media containing different amounts of glucose, yielded re-

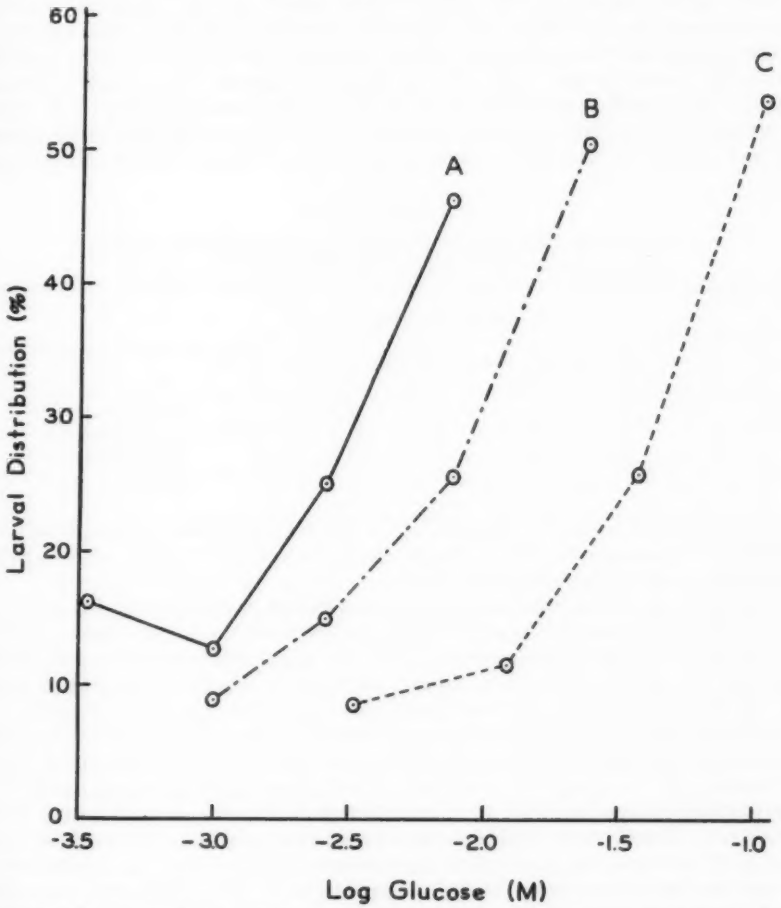

Figure 1. Three experiments (A, B, and C) on the effect of glucose concentration on the distribution of European corn borer larvae among dietary media (four-choice experiments).

sponse curves such as those shown in Figure 1. Over the range of glucose concentrations tested, the larvae tended to concentrate on the highest sugar level available to them. Similar response curves were obtained with sucrose and fructose. The percentage of the larvae established on a given feeding station was apparently independent of the amount of glucose actually present in the medium, but was dependent upon the concentration relative to the concentrations offered in the other media present.

Early in the investigation it became obvious that interpretation of response data would be dependent upon an understanding of the mechanisms by which the larval population became distributed among the feeding stations in proportion to the relative sugar content of the media. Observations on the behavior of newly hatched borer larvae showed that they were initially attracted to the agar-based media, even when the media were devoid of sugars or other nutrients. Such behavior was probably the result of a hydrotactic response. It was also observed that the larvae were quite active up to at least 24 hours after hatching and tended to wander from feeding station to feeding station. They displayed much greater tendency to wander away from stations low in sugar than from those containing

TABLE II

The effect of time of observation on determinations of the influence of glucose on the establishment of newly hatched European corn borer larvae

Time after eclosion (hr.)	Larval establishment		Statistical significance of difference
	control—no glucose (%)	glucose 0.004 *M* (%)	
2	49.3	50.7	not significant
6	29.1	70.9	highly significant
12	28.6	71.4	highly significant

high sugar levels, however. Because of the larvae's sensitivity to light, it was not possible to determine by direct observation the exact influence of sugar concentration on the duration of larval feeding.

On the basis of the observations on larval behavior and the relative nature of the experimental data, the hypothesis that the length of time that a larva remains at a given feeding station is in proportion to the sugar concentration in that station was tested experimentally. Experiments were run in which the effect of the presence or absence of glucose on the distribution of the larvae between two media was measured at different times after the eggs hatched. The results of such two-choice experiments are shown in Table II. The data show that the initial larval distribution between the media was random. By six hours after hatching, the random distribution had been replaced by a non-random distribution in which most of the larvae were found on the feeding stations containing glucose. Although the percentage distribution remained materially constant after six hours, the larvae tended to wander intermittently up to well over 24 hours. At about six hours after hatching, the larval distribution had reached an equilibrium, in which the number of larvae vacating a given feeding station per unit time was equal to the number

arriving from other stations. If the ratios of larval distribution among the media are directly related to the ratios of the average times the larvae remained on the different media, it should be possible to reproduce the experimental data by calculating the equilibrium in time. Table III shows such a calculated distribution equilibrium. Very good agreement between observed and calculated data was obtained. It is apparent that response data, such as shown in Figure 1, represent larval distributions at equilibrium. The percentage distribution of the larvae at the point of equilibrium must be relative to the single experimental variable of sugar concentration, and the distribution data are in agreement with the hypothesis that the response data measure the ratios of the average larval feeding times on the different sugar concentrations.

The general relationship between dietary sugar (glucose) concentration and duration of larval feeding was estimated by the assumption of arbitrary time units in which the average larval feeding time on a just subliminal concentration (0.001 *M*) of glucose was taken as one feeding time unit. Feeding time values were then calculated from larval distribution ratios obtained in a series of two- and four-choice experiments. The regression of feeding time on glucose concen-

TABLE III

Comparison of larval distribution observed in a four-choice experiment with calculated distribution equilibrium

Feeding stations:	A	B	C	D
Larval distribution *observed* (%):	13.0	17.5	27.5	42.0
Distribution ratio (DR) *observed*:	1.00	1.38	2.15	3.38
Larvae leaving station per unit time (1/DR×100) *calculated* (%):	100	72.5	46.5	29.6
Larval distribution at equilibrium *calculated* (%):	12.7	17.5	27.6	42.2
Larval distribution difference (*observed—calculated*):	0.3	0.0	−0 1	−0.2

tration obtained by this method is shown in Figure 2. The value of the feeding time unit, as here defined has not been accurately determined, and the best approximation now possible is that it lies between 20 and 45 minutes. Its value in conventional time units is of little importance, as it will, undoubtedly, change with varying experimental conditions.

The importance of feeding time in the response of insects to sugars is indicated by the published results of several other workers. Kunze (1927) found that the duration of feeding by honey bees could be used as an index of the acceptability of sugar solutions to this insect. Weis (1930) reported that the length of time of uninterrupted feeding was proportional to the sugar (sucrose) concentration of the liquid fed upon by the butterfly *Pyrameis atalanta* L. The volume of liquid ingested per feeding by the honey bee was observed to be directly proportional to the dietary concentration of sucrose (Von Frisch, 1935). Dethier and Rhoades (1954) described certain relationships between the volumes of liquid ingested by adult blowflies and the sugar content of the liquids employed. Using adult blowflies, Frings and Frings (1946) demonstrated that the volume ingested is directly proportional

to the duration of feeding. The tendency for an organism to concentrate its feeding on nutritional substrates high in sugar content has been termed *saccharotrophism* (Beck, 1956b).

From the results presented above, it is apparent that saccharotrophism in the European corn borer, and probably in some other insects, is the influence of sugar concentration on duration of feeding. The experimental data constitute quantita-

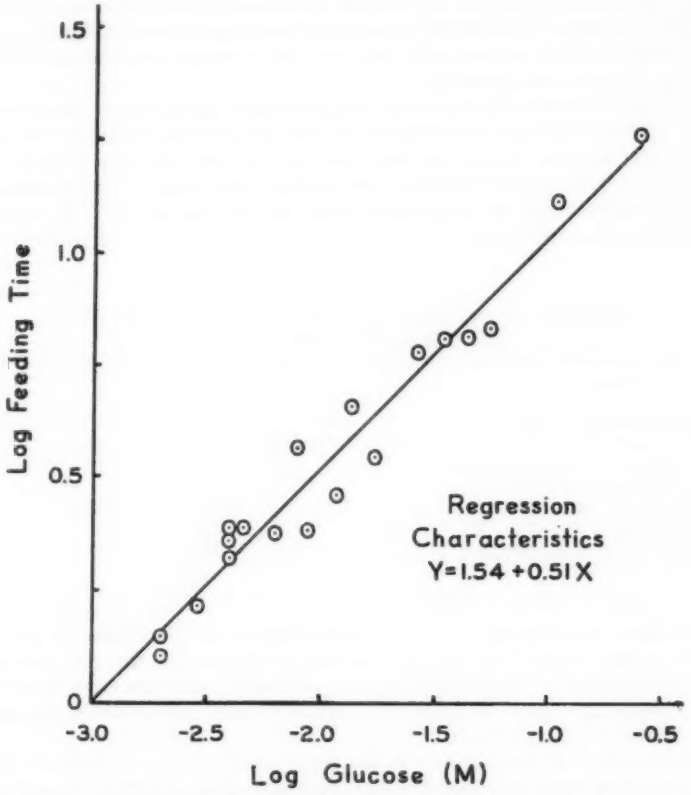

FIGURE 2. The effect of glucose concentration on the duration of feeding by newly hatched larvae of the European corn borer.

tive measurements of the intensity of the saccharotrophic response. The data are not a measure of the proportion of the insect population responding to the sugar concentrations tested. Weis (1930) questioned the reliability of using feeding duration as a quantitative measure of acceptability because of the uncontrolled variables of age, water balance, and nutritional history of the experimental animals. In the present study these factors were standardized by the use of newly-hatched

and previously unfed larvae, and under these conditions the European corn borer larvae employed represented a nearly ideally uniform experimental population.

Bimodal response. During the course of running a series of four-choice feeding experiments designed to allow an estimation of the feeding acceptance thresholds for glucose, sucrose, and fructose, response curves similar to curve A of Figure 1 were frequently obtained. Such curves, in which the response to the lowest sugar concentration was higher than to the next higher concentration, were at first considered to be the result of the statistical vagaries of the experimental method. As more data were obtained, it became evident that responses of this nature were consistently observed in any experiment which included two sugar concentrations somewhat below the apparent acceptance threshold. This phenomenon was investigated in greater detail by means of a series of two-choice experiments in which one of the pair of diets used was devoid of sugars and the other contained a known concentration of glucose. The results are shown in Figure 3. Each point shown in this figure is the mean of the results of from three to five experiments, each of which was made up of eight replicates. Statistical analyses of the data showed that the response to the diets containing sugar was significantly different from the response to the control diets at all sugar concentrations except 0.0001 *M* ($\log = -4.0$) and 0.001 *M* ($\log = -3.0$). At concentrations below 0.001 *M* but above 0.0001 *M*, the larvae tended to concentrate their feeding on the control diets (no sugar) in preference to the diets containing such low levels of sugar. Although the response differences were small, they were reproducible and statistically significant. These data indicate a bimodal response to glucose concentration. At concentrations above an apparent threshold of approximately 0.001 *M*, the feeding response (time) was directly proportional to the sugar concentration and the data are consistent with the Weber-Fechner law. The larval reaction toward concentrations lower than 0.001 *M* was opposite in sign, in that it reduced the feeding period below that elicited by diets containing no sugar. The threshold for the negative response appeared to lie between 0.0001 and 0.0002 *M*. The negative response toward concentrations below the threshold for a positive feeding response was observed in experiments with fructose and sucrose, as well as with glucose. It is therefore apparent that it is not a reaction associated with glucose only. Different manufacturer's lot numbers of glucose (A.C.S. specifications) gave identical results, so the reaction was not a response to the purity of the sugar preparations.

The physiological mechanisms underlying the observed bimodality have not been determined. The data do not necessarily imply a bimodality of response of sugar-sensitive receptor organs, as the present investigation has dealt with only behavioral aspects of the problem. The data show that: (1) the response of European corn borer larvae to different concentrations of sugars is measurable and can be expressed quantitatively in terms of the effect of sugar concentration on the duration of larval feeding. (2) As the sugar concentration is increased above a threshold value lying between 0.0001 *M* and 0.0002 *M*, there is a proportionate increase in the duration of feeding. (3) The response data show a bimodal characteristic in that larval feeding on diets containing low suprathreshold concentrations of sugar is of shorter duration than on diets containing subthreshold amounts of sugar. The following interpretation of these findings is offered, although its speculative nature must be recognized. The duration of feeding is probably related

to the time required for the insect to become adapted to the diet. When in contact with a diet containing no sugar (or a subliminal concentration), stimulation of sugar receptors does not occur, and these sense organs can play no role in determining feeding duration. When they are stimulated, however (as by a suprathreshold sugar concentration), feeding maintenance is dominated by the impulses they feed into the nervous system. Such impulses cease as the larva becomes

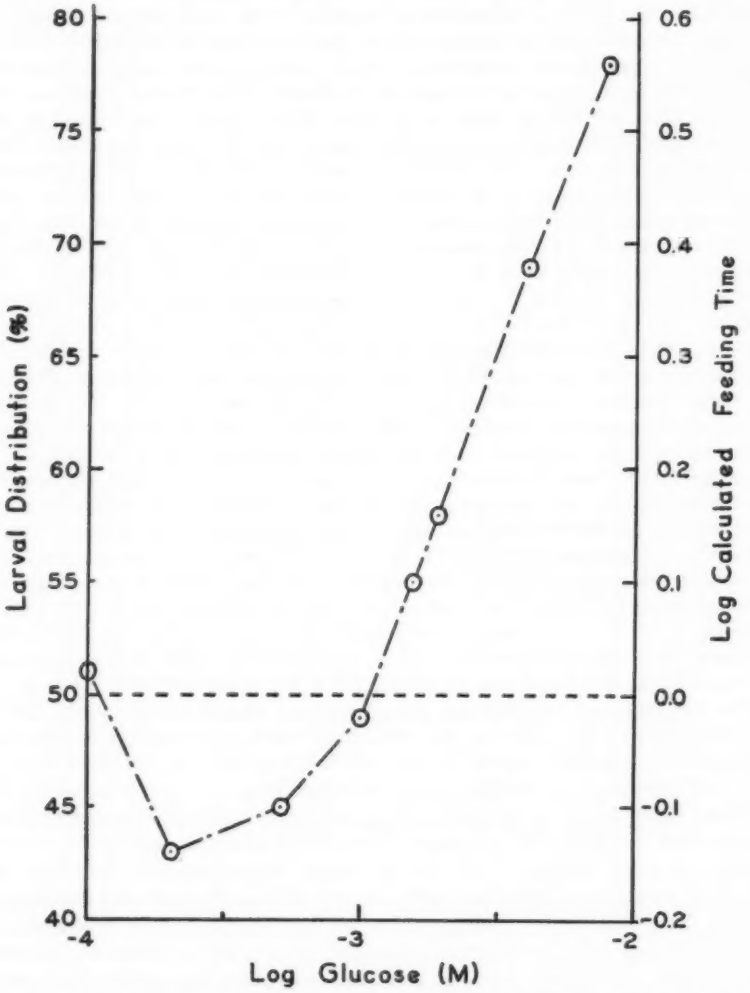

FIGURE 3. Feeding response spectrum of European corn borer larvae for glucose. Two-choice experiments. Plotted data do not include responses to control (no sugar) media.

adapted to the diet, and the behavior pattern undergoes a shift. The lower the sugar level, the more rapid the adaptation, and the shorter the feeding period. In the absence of supra-threshold sugar concentrations, duration of feeding must be determined by other factors. A bimodal response would be obtained experimentally under conditions where duration of feeding is more sensitive to sugar than to alternative factors influencing behavior in the absence of sugars.

A bimodal response to low sucrose concentrations has been reported in human tests by Neuman (1944). Working with human subjects, she found that the quality of sensation caused by sucrose was dependent upon intensity of the stimulus. Her subjects reported that the perceived sensations from sucrose solutions near the threshold level of concentration may be a complex of sweet, bitter, and/or sour. Samuel Renshaw (personal communication) determined human taste spectra for sucrose and found that at the lowest threshold, sucrose gives rise to a smooth tactual sensation rather than to a sweet taste. As the concentration is progressively increased, the taste is bitter, then bitter-sweet, and finally purely sweet. Although it is not possible to determine the quality of sensation produced in an experimental animal, the response data obtained by use of European corn borer larvae show a response spectrum to sugars that is strikingly parallel to the human taste spectrum for sucrose.

The writer is pleased to acknowledge his indebtedness to Mr. E. T. Kaske for technical assistance in conducting the experiments, and to Professors V. G. Dethier and S. Renshaw for their cooperation and review of the manuscript.

Summary

1. A study of the feeding behavior of newly hatched, previously unfed larvae of the European corn borer has shown that dietary sugar concentration has a pronounced and quantitatively measurable effect on such behavior.

2. The larvae tended to prolong their feeding in proportion to the dietary sugar concentrations.

3. The responses to sugar concentrations above a positive response threshold of about 0.001 *M* were in agreement with Fechner's law over the concentration range tested. There was a small but significant negative feeding response toward sugar concentrations below 0.001 *M* and down to 0.0002 *M*.

4. The experimental results obtained indicate that this organism displays a bimodal response to glucose, sucrose, and fructose, in which the modes differ in sign and are dependent upon the concentrations employed. The response spectrum of the insect toward the sugars tested appears to be parallel, if not directly comparable, to the human taste spectrum for sucrose.

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A HISTOLOGICAL AND CYTOPHOTOMETRIC STUDY OF THE EFFECTS OF X-RAYS ON THE MOUSE TESTIS[1]

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Studies of Bergonié and Tribondeau (1904), Regaud and Blanc (1906) and others focussed attention on irradiation-induced changes in reproductive organs and gametes of various organisms. Their findings indicate that spermatogonia are the most x-ray-sensitive components of the seminiferous epithelium and that, following the decrease in numbers of spermatogonia, the other cell types disappear in the order of their development. The topic has been the further subject of numerous publications and the literature has been reviewed by Heller (1948).

Investigations of the nature of x-ray-induced sterility in the male mouse have been carried out by Snell (1933) and by Hertwig (1938). Recent reviews by Kaufmann (1954) and Russell (1954) have dealt with radiation-induced changes of cytological and genetic interest.

Recently, emphasis has been given to quantitative histological studies of irradiated testes. Studies have been carried out by Eschenbrenner, Miller and Lorenz (1948), Eschenbrenner and Miller (1950), Fogg and Cowing (1951), Barrow and Tullis (1952), Shaver (1953) and Oakberg (1955). Investigations have been facilitated by the histological scoring procedure developed by Chalkley (1943) and by the cytological studies of Roosen-Runge and Giesel (1950), and of Leblond and Clermont (1952).

The present approach differs from the above in that it represents the combination of a quantitative histological method together with cytophotometry, thus affording data with respect to the DNA content of irradiated tissues as well as changes in the frequency of cells.

Materials and Methods

Two inbred strains of mice, differing in their sensitivity to mouse typhoid, were used in these experiments. The animals chosen were 58-day old males of strains BALB/Gw (hereinafter designated as Ba) and S. The animals were irradiated in plastic tubes and were exposed to a dose of 320 r (250 pkv, 30 ma; filtration 0.25 mm. Cu, 1 mm. Al; anode-target distance 47.5 cm., dose rate 430 r/min.). The irradiation was delivered to the pelvic region only, the rest of the body being shielded with lead.

Control and irradiated animals were killed at 1, 8 and 24 hours, 3, 5, 10, 16 and 28 days after irradiation. From each animal one testis was removed, quickly freed of associated tissues, rolled on filter paper, weighed on a Roller Smith torsion bal-

[1] Journal paper No. J-2875 of the Iowa Agricultural Experiment Station, Ames, Iowa. Project No. 1187. This work has received assistance from Contract No. AT(11-1)107 from the Atomic Energy Commission.

ance and dropped into fixative. The other testis was removed and treated in a similar manner, and was later used for dry weight determinations. The testis in fixative was then cut into several pieces. Very few minutes elapsed from the time each animal was killed until the testis removed into fixative was subdivided.

In the case of the one-hour controls, kidney tissue also was fixed and later served as a control tissue for the Feulgen reaction.

Fixation

All tissues were fixed in Carnoy's acetic-alcohol (1:3) for three hours, washed in several changes of absolute alcohol, cleared in benzene and embedded in 56–58° C. Tissuemat. Sections were cut at 7 and at 10 μ. For photometric purposes, sections from all animals of one strain were mounted on the same slide together with sections of kidney obtained from a Ba control animal.

Staining

The desoxyribose nucleic acid (DNA) content of nuclei was visualized by means of the Feulgen reaction. The reagent was prepared according to the method of Stowell (1945) and sections were stained for two hours at room temperature following hydrolysis (12 minutes) in 1 N HCl at 60° C. Some Feulgen preparations were lightly counterstained with fast green and used for cytological studies. Unhydrolyzed slides were immersed in the Feulgen reagent and served as controls for photometric purposes.

Other slides were stained with a mixture of safranin and fast green as described by Bryan (1955). These preparations were used for routine cytological and histological studies as well as for the quantitative scoring procedure described below.

Additional slides were stained by means of the periodic acid-Schiff (PAS) reaction. The reagents were prepared according to McManus (1948) and the slides were stained as described by Bryan (1954), methyl green being used as a counterstain. This procedure was used, in the earlier phases of this work, as an aid in the identification of the developmental stages of the seminiferous epithelium, after the manner described by Leblond and Clermont (1952). It should be mentioned, however, that the stages of development of the seminiferous epithelium as described for the rat do not entirely correspond to the stages observed in the mouse.

Photometry

Measurements of the DNA-Feulgen complex were made with an apparatus essentially the same as that described by Pollister (1952) and others. A tungsten filament lamp (6 volt, 18 amp.) was used as a light source and the spectral lines isolated by means of a Perkin-Elmer monochromator equipped with appropriate accessory optics.

It was necessary to compare data obtained from several slides. Therefore, sections of control kidney were used as a standard to check on the reproducibility of the Feulgen reagent. Means of the DNA-Feulgen content of kidney nuclei obtained from the different slides did not differ significantly from each other.

Transmittance data were obtained by the "plug" method described by Swift

TABLE I[1]

Frequency of cell types at various times after 320 r of x-rays

Stage and strain		1 hour	8 hours	1 day	3 days	5 days	10 days	16 days	28 days
Spermatogonia in interphase	Ba	102.6	110.3	91.0	1.1	9.2	10.4	50.1	161.3
	S	107.8	101.5	79.2	2.2	0.0	3.3	37.7	187.6
Spermatogonia in mitosis	Ba	82.9	26.8	65.9	4.9	12.2	9.8	56.1	173.2
	S	102.6	11.5	21.5	0.0	0.0	2.3	26.4	222.8
Spermatocyte chromatin	Ba	96.3	98.3	109.3	116.7	92.4	64.8	10.0	110.5
	S	96.3	101.6	107.6	112.1	97.2	82.8	0.0	122.2
Spermatid nuclei	Ba	95.1	97.0	87.3	93.4	122.5	144.4	126.4	49.8
	S	111.8	95.5	99.7	106.0	132.5	144.1	149.5	23.1
Sperm heads	Ba	112.8	105.2	113.8	134.7	146.6	228.4	369.7	51.6
	S	89.6	107.6	97.0	112.2	132.4	246.7	358.3	63.4
Sertoli nuclei	Ba	104.9	110.0	97.0	72.8	96.5	125.4	139.1	115.1
	S	99.3	85.9	104.7	113.4	125.5	160.4	125.9	136.6

[1] Values expressed as per cent of controls.

(1950), and were converted into extinction values ($\log_{10} {}^1/T$). From the extinction data, the amounts of the DNA-Feulgen complex were computed as described by Swift (1950). In most cases the nuclei selected for measurement purposes were located beforehand and their location indicated on a camera lucida map.

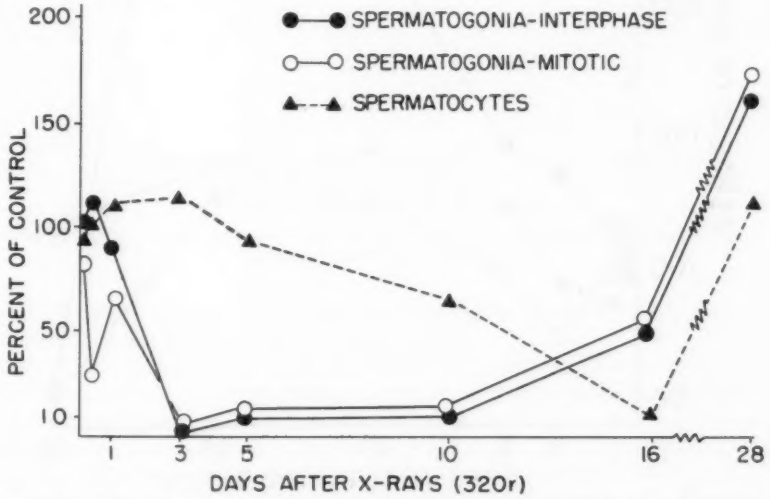

FIGURE 1. Strain Ba. Incidence of spermatogonia and spermatocytes at different times following 320 r of x-rays.

Scoring of cells

Estimates of the relative areas of the seminiferous tubules occupied by each stage of spermatogenesis were obtained by means of the quantitative scoring procedure developed by Chalkley (1943). The manner in which this procedure was employed was based on a similar application by Eschenbrenner, Miller and Lorenz (1948) but with minor modifications.

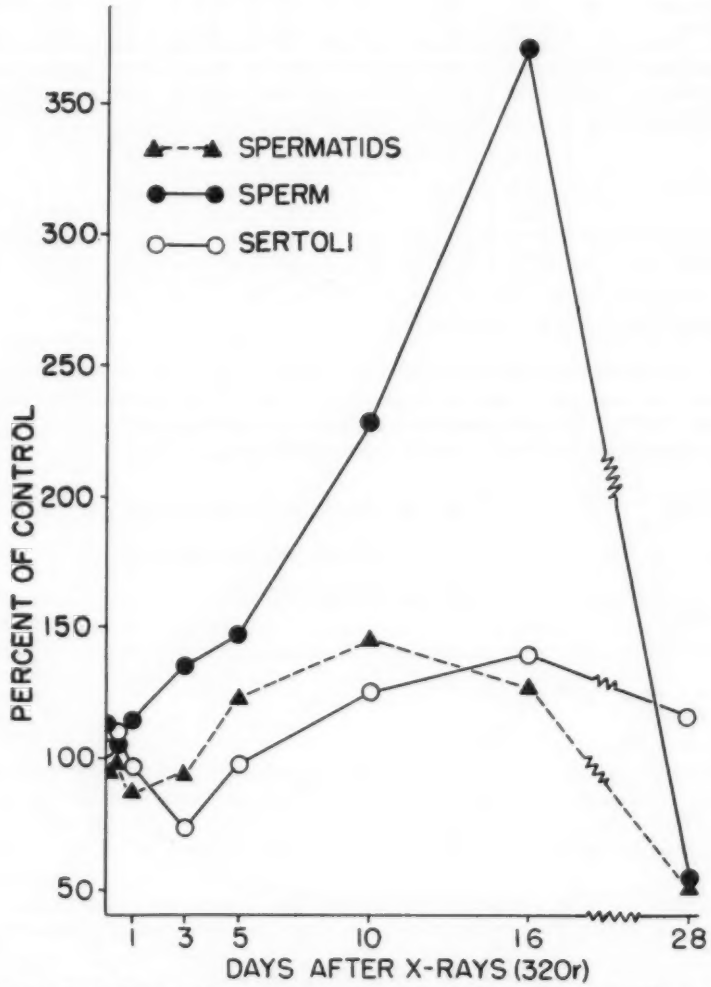

FIGURE 2. Strain Ba. Incidence of spermatids, sperm and Sertoli nuclei at different times following 320 r of x-rays.

Nuclei were recorded as belonging to the following classes: interphasic spermatogonia, dividing spermatogonia, spermatocytes, spermatids, sperm and Sertoli nuclei. Eschenbrenner *et al.* did not record "hits" on Sertoli nuclei and recorded only spermatids possessing spherical nuclei and only those sperm heads evincing "well defined dorsal fins." In the present work, all stages of spermatid development and of sperm maturations were recorded. On account of these differences in procedure, the results of Eschenbrenner *et al.* and those reported here are not strictly comparable.

Results

A. Histological studies

The histological scoring data are summarized in Table I and are presented in graph form in Figures 1–4. In Table I the data pertaining to both strains are

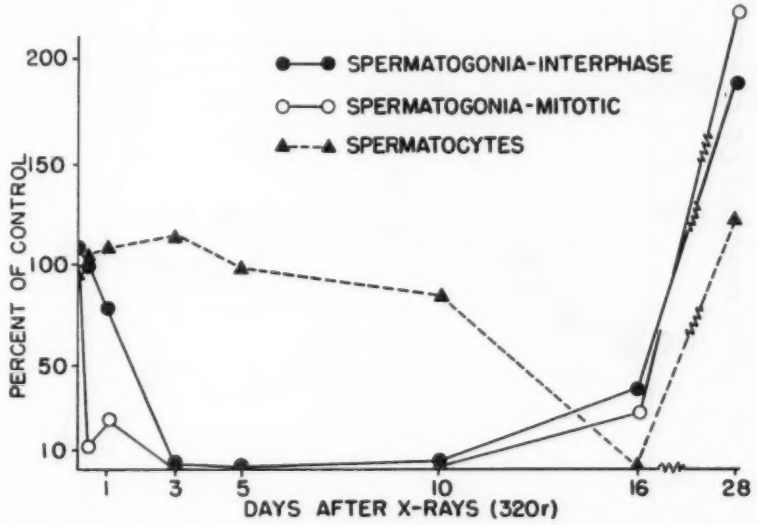

FIGURE 3. Strain S. Incidence of spermatogonia and spermatocytes at different times following 320 r of x-rays.

presented together in order to facilitate their comparison. All values in this table are expressed in terms of per cent of control values.

These data indicate that with the exception of the dividing spermatogonia, there is no pronounced change in the cellular composition of the seminiferous epithelium during the first 24 hours following irradiation. During the period of 1–3 days after irradiation there occurs a marked decline in the area occupied by interphasic spermatogonia. Some regeneration of spermatogonia is apparent by 10 days and is well underway by 16 days. Dividing spermatogonia, on the other hand, undergo a rapid decrease during the period of 1–8 hours after irradiation. At 24 hours,

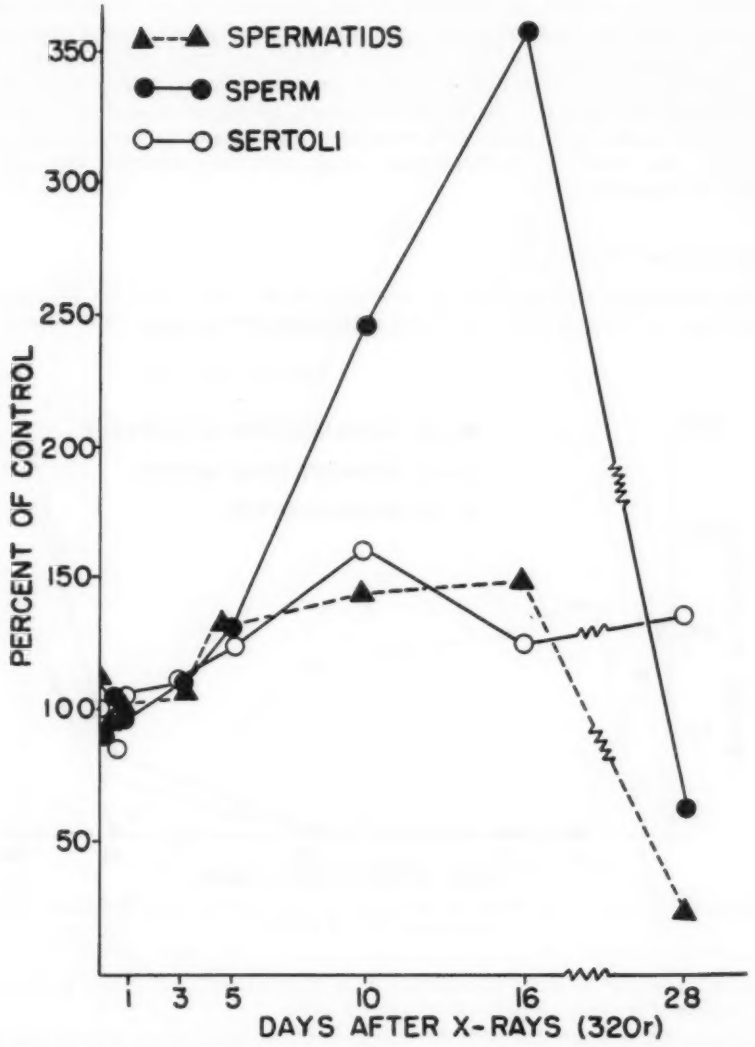

FIGURE 4. Strain S. Incidence of spermatids, sperm and Sertoli nuclei at different times following 320 r of x-rays.

the area occupied by these cells shows a moderate increase (from 26.8% of the control value at 8 hours to 65.9% of the control value at 24 hours in strain Ba, and from 11.5% to 21.5% in the case of strain S). This increase is only a transient feature since by three days the area occupied by dividing spermatogonia has further

declined to a level of less than 5% of the control value. Thereafter, the dividing spermatogonia show essentially the same behavior as the interphasic cells.

During the course of the histological studies it was found that the seminiferous epithelium of both control and irradiated animals contained cells in various stages of degeneration. The frequency of such cells appeared to be greater in the irradiated material taken from the animals killed during the early part of the post-irradiation period. Accordingly, the pertinent sections were analyzed by means of the Chalkley procedure and the data (expressed as percentages) obtained, from both strains, are presented in Table II.

B. DNA-Feulgen

The data obtained are summarized in Tables III and IV. The values represent the mean amounts of the DNA-Feulgen complex (in arbitarary units) together with

TABLE II

Spermatogonial and non-spermatogonial necrosis after 320 r of x-rays

Strain	Time after irradiation	% spermatogonial necrosis	% non-spermatogonial necrosis	% total necrosis[1]
	Control			
Ba		3.75	2.07	2.27
S		4.03	2.52	2.76
	1 hour			
Ba		3.40	2.99	3.05
S		5.76	2.37	2.83
	8 hours			
Ba		9.71	2.24	2.98
S		8.80	1.68	2.56
	24 hours			
Ba		7.41	2.26	2.79
S		4.55	2.21	2.36

[1] $\frac{\text{No. of necrotic cells}}{\text{total cells scored}} \times 100.$

their respective standard errors. Nuclei possessing the diploid amount of DNA are designated as Class II nuclei, those with twice this amount Class III, and Class IIa represents nuclei possessing an amount of DNA intermediate between these levels.

The range of values for the DNA classes was determined as follows. In the case of the control kidney tissue, the highest value obtained was found to be about 50% higher than the lowest (values ranged from 1.78 units to 2.69 units). The Feulgen-stained kidney nuclei have a more uniform appearance than the spermatogonial nuclei and consequently the spread of values in the latter is somewhat larger with amounts ranging from 1.91 units to 2.94 units. As an approximation to the range expected for Class III nuclei these values may be doubled, yielding a range of from 3.82 to 5.88 units. In actual fact the values for these nuclei were

found to range from 3.9 units to 5.6 units but with most of the nuclei distributed over a range of 4–5 units. Thus the spread of values is rather lower than expected. It may be accounted for on the basis of distributional error since many of these nuclei (which are slightly larger than Class II nuclei) show clumping of the stained material. Those values falling between the upper limit of Class II and the lower limit of Class III were assigned to Class IIa.

C. Other observations

The irradiated material was examined for indications of damage to the chromosomes. No indications of extensive damage, at the cytological level, were seen.

TABLE III

DNA-Feulgen content of spermatogonia, strain Ba

Tissue and treatment	Class	No. of nuclei	DNA-Feulgen content
Kidney—control	II	54	2.15±0.03
Testis—control	II	96	2.42±0.04
	IIa	71	3.51±0.04
	III	46	4.52±0.06
Testis—320 r 1 day	II	24	2.57±0.05
	IIa	24	3.44±0.05
3 days	(No nuclei suitable for measurement)		
5 days	II	31	2.52±0.05
	IIa	11	3.30±0.09
	III	1	4.32
10 days	II	29	2.52±0.05
	IIa	12	3.36±0.06
	III	7	4.26
16 days	II	52	2.28±0.07
	IIa	24	3.41±0.05
	III	4	4.16
28 days	II	38	2.54±0.05
	IIa	35	3.50±0.05
	III	7	4.30

The frequency of anaphase figures showing one or more bridges appeared to be very low. During the early period of regenerative activity "nests" of spermatogonial cells were occasionally seen containing one or (rarely) more anaphase bridges. The underlying mechanism responsible for this is not understood; it would appear that in some instances the irradiated spermatogonia must have divided at least once before the chromosomal aberrations showed up.

Other instances of cellular (or nuclear) damage were also observed. Thus, giant nuclei were found in some tubules of the irradiated material. These nuclei

made their appearance at about 5 days after irradiation and persisted until 16 days, reaching an apparent high point at 10 days. In addition a few of the tubules examined were found to contain cysts composed of 4 to 8 spermatogonial-like nuclei. The fate of these structures is unknown.

At 16 days after irradiation, spermatozoa occupy about 40% of the area of the seminiferous epithelium. It is of interest that some sperm heads present an abnormal appearance. In contrast to the controls, where the Feulgen-positive material (DNA) is distributed in a relatively homogeneous manner, the abnormal sperm

TABLE IV

DNA-Feulgen content of spermatogonia, strain S

Tissue and treatment	Class	No. of nuclei	DNA-Feulgen content
Kidney (Ba)—control	II	30	2.11±0.04
Testis—control	II	40	2.46±0.05
	IIa	47	3.41±0.04
	III	13	4.29±0.07
Testis—320 r 1 day	II	37	2.66±0.04
	IIa	46	3.34±0.03
	III	2	4.73
3 days	(No nuclei suitable for measurement)		
5 days	(No nuclei suitable for measurement)		
10 days	II	21	2.36±0.09
	IIa	6	3.57
16 days	II	44	2.48±0.05
	IIa	24	3.37±0.04
	III	2	4.22
28 days	II	53	2.50±0.05
	IIa	25	3.26±0.03
	III	2	4.20

heads have a "bubbly" structure. Such sperm may have been derived from irradiation-damaged spermatids or, possibly, spermatocytes.

DISCUSSION

In addition to histological and genetic approaches, the effects of irradiation on biological systems have been studied from the biochemical viewpoint with the aid of tracer techniques. Thus Holmes (1947) used P^{32} in his studies of the effect of x-rays on the synthesis of nucleic acids in the rat Jensen sarcoma. He found that irradiation markedly inhibited the uptake of P^{32} into the DNA fraction of the tumor. Studies by Hevesy (1949) have suggested that the delay in mitosis following irradiation is related to the marked inhibition of DNA synthesis, as

measured by C^{14} uptake into DNA purines. It has been shown also, by Abrams (1951), using glycine-1-C^{14}, that irradiation greatly reduces the incorporation of radioactive carbon into the DNA and PNA purines of rat and rabbit intestine, whereas the synthesis of proteins is relatively unaffected. Similarly, Smellie *et al.* (1955) also find marked inhibition of P^{32} incorporation into the nucleic acids of normally mitotically active rabbit tissues following irradiation with 1000 r of x-rays.

Recent work by Klein and Forssberg (1954), using the Ehrlich mouse ascites tumor, indicates that 1250 r of x-rays completely suppresses mitotic activity for a period of 16 hours without any increase in the cell death rate. A linear increase of cell volume with time followed the irradiation treatment. By chemical means, these workers showed that PNA and total nitrogen per cell increased at nearly the same rate as cell volume; DNA, however, did not show this behavior. Later studies by Forssberg and Klein (1954), using glycine-2-C^{14}, point in the same direction, that is, that irradiation brings about a marked decline in DNA synthesis, and, simultaneously, inhibits mitotic activity.

With the aid of a cytophotometer it is possible to measure the DNA-Feulgen content of individual spermatogonial nuclei in interphase. The data presented in Tables III and IV show that in the controls three classes of nuclei are present—those possessing the diploid amount of DNA-Feulgen complex (Class II), the tetraploid amount (Class III) and nuclei containing an intermediate amount of DNA (Class IIa). This latter group is representative of those nuclei engaged in the reduplication of their chromatin content in preparation for the next division, whereas Class III represents spermatogonial nuclei in which synthesis is complete, while the changes involved in the initiation of prophase have not yet occurred.

Irradiation brings about an inhibition of DNA synthesis, as measured by the uptake of radioactive precursor molecules. Hence, after irradiation, it would be expected that those nuclei which had been prevented from reduplicating their DNA content would be unable to enter mitosis. The net result should be loss, through mitosis, of the Class III nuclei. During the recovery process, DNA synthesis recommences and Class III nuclei should be produced. The DNA-Feulgen data presented in Tables III and IV are in agreement with these ideas. Thus, in the control measurements, Class III nuclei are relatively abundant, whereas shortly after irradiation, they are either absent or very much reduced in number.

It is not possible to determine directly whether or not the first spermatogonia to enter mitosis, after irradiation, are those which, though possessing the requisite amount of chromosomal material, had been prevented from dividing. However, the cytophotometric data strongly suggest that this is the case. The reason is that if such nuclei were prevented from entering mitosis then it should be expected that at 24 hours after irradiation a significant number should be present. The data presented in Tables III and IV show that no Class III nuclei were present in the sample obtained from strain Ba and only two such nuclei (2.4% of the sample) were encountered in the case of strain S.

It might be expected that at 28 days after irradiation, when the frequency of spermatogonial nuclei is much higher than in the controls, that a large proportion of these nuclei should prove to belong to Class III. However, the dry weights of irradiated testes at this time comprise only 34–47% of the control weights. These facts, together with the photometric data, may be taken to suggest that the high

rate of mitosis is of a compensating nature and that the spermatogonia enter mitosis immediately after the reduplication of the chromatin is completed.

As shown by Bullough (1948), mitotic activity in the mouse testis remains about constant and at a high level throughout the day. According to the previously mentioned tracer studies, irradiation should bring about a marked decline in the mitotic rate of the seminiferous epithelium (and hence in the area of the tubules occupied by mitotic figures). Reference to Table I and Figures 1 and 3 indicates that at one hour after irradiation, the area of the tubules occupied by dividing spermatogonia is not very different from the control value in the case of strain S, and somewhat lower in the case of strain Ba. By 8 hours, the level has fallen to about 27% of the control in the case of strain Ba, and to 12% in strain S. However, at the present time one cannot rule out the possibility that the mitotic activity has undergone a more rapid decline prior to 8 hours, by which time some regeneration is underway. That this is the case might, perhaps, receive some support from the findings that the one-day values show a substantial increase over the 8-hour levels (from 26.8% of the control value at 8 hours to 65.9% of the control value at 24 hours, or an increase of 140%, in the case of strain Ba; from 11.5% to 21.5%, or an increase of 87%, in the case of strain S).

The duration of mitotic delay (temporary inhibition) as a function of dose has been investigated by Canti and Spear (1929), Henshaw (1932, 1940) and others. (See Lea, 1955, for a discussion of this point.) While the period of delay, for a given dose, varies with the different organisms used, the findings allow the generalization that, for 300 r, recovery would be expected to commence at about 6–8 hours after irradiation. The results reported here for the mouse testis are in fair agreement with this estimate.

The stage at which cells are most sensitive to irradiation has been investigated by Spear (1935), Carlson (1940, 1941, 1954), Creighton (1941) and others. Creighton (1941) has reported the most sensitive stage for grasshopper spermatogonia to be three hours prior to anaphase, that is, at late interphase or early in prophase. The results of Carlson (1941), working with grasshopper neuroblasts, indicate the critical stage to be in prophase, while Spear (1935) concludes from his tissue-culture experiments that late interphase is the most sensitive stage. The work of Henshaw (1940) and others on *Arbacia* eggs suggests prophase to be the most sensitive stage.

The differences noted above are probably not fundamental, but merely represent differences due to the conditions of the experiments in question. The main point is that the most sensitive stage appears to be that part of the mitotic cycle lasting from late interphase to early prophase. Irradiation at or immediately prior to this stage causes the cells to be held up, the duration of this mitotic delay being dependent upon the dose received.

At the time of irradiation, the further away a cell is from this critical stage in its development, the less it is retarded. Similarly, cells which have progressed beyond this stage, at the time of irradiation, do not undergo inhibition of mitosis. These observations afford a partial explanation for the present findings—that the rise in the mitotic rate during the period of 8–24 hours after x-rays is followed by a further and more extensive decline.

In the testis, blockage of spermatogonial mitoses would be expected to result

in maturation depletion of the tubules since the spermatogonial cells would be unable to undergo the necessary divisions to renew the proportion of cell types lost (through maturation). The present results confirm this view.

The suppression of DNA synthesis produced by irradiation appears to be induced in cells which were irradiated in interphase since cytophotometric investigations by various workers (see for instance Swift, 1950 and Bryan, 1951) indicate that reduplication of the DNA content, in preparation for the next division, takes place during this phase of the mitotic cycle. Further support for this conclusion may be drawn from the work of Gentcheff and Gustafsson (1939). The findings of these authors suggest that irradiation-induced inhibition of mitosis is caused by the prevention or delay of chromosomal reduplication and consequently the sensitivity of cells to x-rays is correlated with the time of chromosome reproduction.

It is evident that irradiation of cells and tissues brings about specific effects at different levels of organization. Thus at the molecular level there are, for example, such effects as the denaturation of SH-group-containing proteins, and disturbances of the systems responsible for the synthesis of the nucleic acids. At the cytological level, diverse chromosomal observations are encountered as well as effects resulting in the prolongation of prophase (or late interphase). Then there appear to be various latent cellular effects which become evident at a later date—in some instances one or more cell generations later.

It should be expected that a number of cells would receive lethal doses of radiation resulting in immediate death or death prior to the completion of a division cycle. It then follows that if sufficient spermatogonia are destroyed in this manner, maturation depletion of the tubules would ensue. The data of Oakberg (1955) strongly support this hypothesis. However in the present work, as can be seen from Table II, the proportion of necrotic spermatogonia constitutes only a minor fraction of the total. This work therefore supports the alternative hypothesis; that is, irradiation-induced maturation depletion of the seminiferous epithelium is brought about mainly through the irradiation-induced changes responsible for the inhibition of mitotic activity, with cell death playing only a minor role in this process. These conclusions, based on histological and cytophotometric data, therefore do not support the findings of Oakberg (1955), but instead, offer further confirmation of the findings of Eschenbrenner and Miller (1950).

In conclusion, then, the data presented in this paper, with respect to irradiation-induced maturation depletion of the seminiferous tubules, do not support the spermatogonial cell death hypothesis. Instead the data are consistent with the view that the major effects of irradiation are (a) the suppression of mitosis due to inhibition of DNA synthesis and (b) the suppression of mitosis due to the prolongation of prophase through the disturbance of other physiological and biochemical processes necessary for normal mitotic activity.

Summary

1. Changes in the seminiferous epithelium, induced by 320 r of x-rays, have been analyzed by means of a quantitative histological procedure.

2. With the aid of a cytophotometer, measurements have been made of the DNA-Feulgen content of control and irradiated spermatogonial nuclei.

3. The histological data indicate that irradiation, with 320 r of x-rays, results in a temporary maturation depletion of the seminiferous epithelium. This depletion is brought about through the inhibition of spermatogonial mitosis rather than irradiation-induced spermatogonial necrosis. The frequency of spermatogonia reaches its lowest point by three days after irradiation and regeneration does not begin until after 10 days.

4. The cytophotometric data suggest that irradiation-induced inhibition of DNA synthesis is a contributing factor with respect to the suppression of mitotic activity.

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THE ACROSOME FILAMENT AND SPERM ENTRY IN THYONE BRIAREUS (HOLOTHURIA) AND ASTERIAS[1]

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In a number of sea urchin, starfish and mollusc species, Dan (1952, 1954a and 1954b) and Dan and Wada (1955) have demonstrated that the spermatozoon undergoes a profound reaction when treated in one or another of the following ways: exposure to egg water, alkaline sea water, or the presence of eggs; contact with surfaces such as that of glass, a collodion membrane or the egg. The salient feature of this reaction is the formation of a filament, the *acrosome filament*, which extends from the acrosome region. Among other changes is an altered relationship of principal structures. In the echinoderm species the middle piece tends to become displaced to an excentric position and the base of the flagellum then appears to emerge from between middle piece and head, forming almost a right angle with the long axis of the spermatozoon and hence with the projecting acrosome filament.

Spermatozoa having the acrosome filament and structural relationships of reacted specimens described by Dan have also been observed in *Holothuria atra* (Colwin and Colwin, 1955a), in suspensions of sperm treated with egg water or placed in the presence of eggs. In this species it was demonstrated not only that the acrosome filament becomes associated with the egg proper but also that the filament enters the egg intact, as an integral part of the spermatozoon.

The present paper will show that the spermatozoa of another holothurian, *Thyone briareus*, produce acrosome filaments of striking length when appropriately stimulated. The data are, in addition, quantitative. The behavior of the acrosome filament during sperm entry will be described. Collateral confirmatory information on two species of starfish, *Asterias forbesii* and *Asterias vulgaris*, will be included. Abstracts of some of these observations have appeared previously (Colwin and Colwin, 1955b and 1955c).

Materials and Methods

Thyone briareus. Specimens were kept in running sea water. Eggs and spermatozoa were obtained from 15 females and 18 males which were eviscerated at intervals between May 13th and June 19th, and from 6 females and 6 males which shed naturally between June 16th and 20th, 1955. Evisceration, a common response in *Thyone* (Killie, 1939), was induced by electrical stimulation of individual animals; then when the gonadal areas were compressed by hand, the gonads were shed along with the other organs. All gonads were rinsed at once in filtered

[1] Appreciation is expressed for laboratory facilities during the summer of 1955, provided under a contract of the Office of Naval Research with The Marine Biological Laboratory.

sea water. The testes were then stored "dry." To obtain sperm the tubules were cut once or twice with scissors. The sperm which oozed out was then suspended in filtered sea water and variously diluted. No differences were detected between the active spermatozoa obtained in this manner and those shed naturally. Eggs were gently stripped through the cut ends of individual ovarian tubules. A thin cellular capsule, which did not persist on naturally shed eggs, enclosed the stripped egg and its surrounding jelly. Frequently this capsule could be completely or partially removed by sucking the eggs into a glass syringe through a hypodermic needle of No. 27 bore. Then the needle was detached and the eggs slowly expelled into filtered sea water. Effective egg water was procured from these decapsulated stripped eggs as well as from eggs which had been shed naturally.

The egg of *Thyone* is flattened axially. It appears elliptical in lateral view and somewhat circular in polar view; the diameter of this circle is approximately 250 μ. Near the animal pole a typical holothurian "umbilicus" projects from the egg into the surrounding jelly. The jelly hull is approximately 55 μ thick and contains radial striations, some extending as far as its outer edge. These irregular wavy striations differ markedly from the clearly-defined rigid looking acrosome filaments of entering spermatozoa.

Sections of naturally shed eggs show that the germinal vesicle is not intact, confirming the findings of Ohshima (1925). In the living egg, however, the vesicle often *appears* to remain intact owing probably to persistence of its "residual substance," as noted by Ohshima. Eggs from eviscerated gonads were not sectioned.

Asterias. Germ cells of *A. forbesii* were obtained from freshly collected specimens in May and June, 1955. The gonads were removed from detached arms. Ovaries placed in large dishes of sea water shed copiously. Fresh eggs were removed, rinsed several times and put into large dishes of sea water. Concentrated sperm, collected from testes which had been stored "dry," was suspended and diluted in filtered sea water as needed. A few specimens of *A. vulgaris,* observed on May 14th, were also handled in the manner just described.

Alkaline sea water was prepared by adding sufficient 0.1 N NH_4OH to freshly diluted sea water to bring it to pH 9.2–9.4, as determined by color comparison with thymol blue. In some cases a slightly higher pH was used. The sperm suspensions were then added to this alkaline sea water. The actual pH of these final mixtures was not determined but would obviously be lower than that of the original alkaline sea water.

All photographs and sketches shown were made from living material as viewed with oil immersion objectives. Both bright field and phase contrast microscopes were used.

Observations

I. The acrosome filament

A. *Thyone briareus*

1. *Formation of acrosome filament in treated sperm suspensions.* Spermatozoa were treated with either *egg water* or *alkaline (ammoniated) sea water.* Drops of sea water-suspended sperm were added to drops of either solution and, for controls, to filtered sea water in the same proportions. Some typical results as found in

living preparations are shown in Table I. In all these experiments appropriate treatment of the sperm resulted in the production of acrosome filaments in percentages significantly greater than the controls. The alkaline sea water was a much more effective stimulant than the egg water within the concentrations of the latter used. This is shown strikingly in experiments 6 and 7 in which in each case one original sperm suspension was used with the egg water and the alkaline sea water; in both experiments the egg water was ineffective (too weak?) whereas the alkaline sea water caused 90% or more of the spermatozoa to form acrosome filaments.

TABLE I

Acrosome reaction of spermatozoa of Thyone briareus. Production of acrosome filaments evoked by adding sea water sperm suspensions to egg water or to sea water made alkaline by addition of 0.1 N NH_4OH

Number of experiment	Number spermatozoa counted	% with filaments	Number spermatozoa counted	% with filaments
	Egg water		sea water control	
†EG, SN–1	100	21	100	2
EG, SN–2	52	21	>100	0
EG, SG–3	100	18	100	3
EN, SN–4	104	12.6	100	1
EG, SG–5	73	6.8	100	0
EG, SG–6(a)	111	1.8	††sweep	0
EG, SG–7(a)	100	0	sweep	<1
	Alkaline sea water		sea water control	
EG, SG–6(b)	sweep	>90*	sweep	0
EG, SG–7(b)	sweep	>99*	sweep	<1
EG, SG–7(c)	97	92**	sweep	<1
EG, SG–8	sweep	>90**	sweep	0

Experiments bearing the same number were made simultaneously from the same sperm suspension and have the same control.

† E, eggs; S, sperm; N, naturally shed; G, obtained by removal from gonad.

†† Sweep: extensive examination of area of coverslip by back and forth movement with a mechanical stage.

* pH of alkaline sea water to which sperm was added was slightly higher than 9.2–9.4.

** pH of alkaline sea water to which sperm was added was 9.2–9.4.

2. *Acrosome filaments in untreated sperm suspensions.* Examination of the control figures of Table I makes it clear that spermatozoa with acrosome filaments are sometimes found in a very low percentage in untreated sperm suspensions. Perhaps these result from "contact" stimulation, as Dan has suggested for other species, or from as yet unknown factors.

3. *Acrosome filaments in inseminated cultures of eggs.* The acrosome filament is always found on spermatozoa which are entering eggs. It is also seen on spermatozoa which lie in the jelly but fail to enter the egg; it may (Figs. 2, a and 8) or may not extend as far as the egg surface; its position in the jelly is usually radial, sometimes tangential and occasionally even directed away from the egg surface.

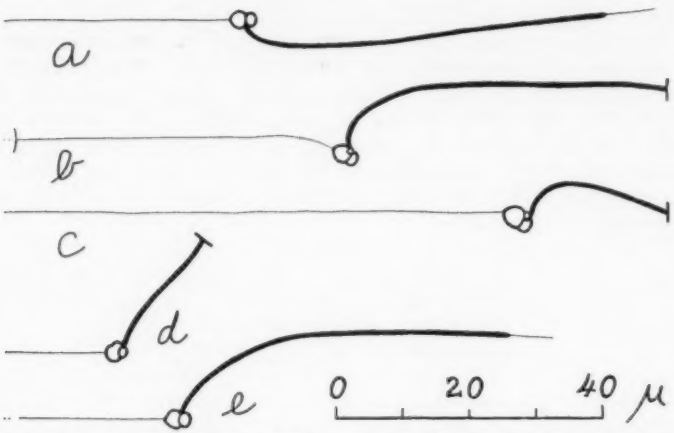


FIGURE 1. Reacted spermatozoa showing acrosome filament, from photographs of living specimens. a–c, *Thyone briareus;* d–e, *Asterias forbesii.* a, in egg water (35 μ); b, at egg surface (48 μ); c, in alkaline sea water (75 μ) (position of distal part of flagellum modified for reasons of space); d, in inseminated culture but directed away from egg (15 μ); e, at egg surface (22 μ).

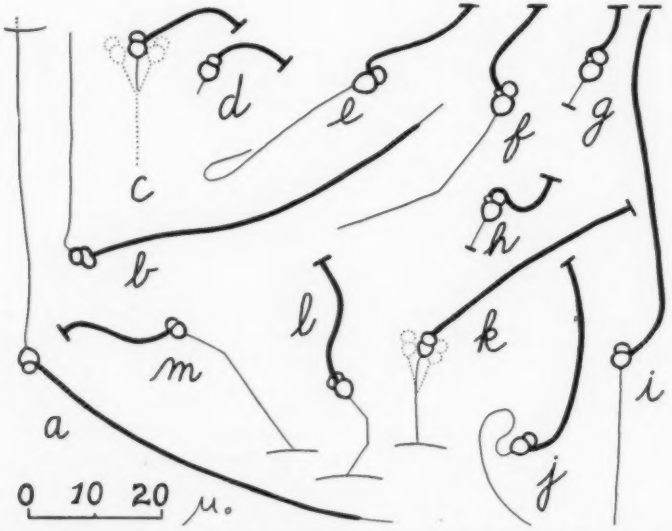


FIGURE 2. Reacted spermatozoa: bending and curving of acrosome filament. From sketches and photographs of living specimens. a–h, *Thyone briareus;* i–l, *Asterias forbesii;* m, *A. vulgaris.* a, at egg (presumed distal portion in dotted line); b, in egg water; c–h, in alkaline sea water. c, oscillating about fixed point (presumed distal part of acrosome filament dotted); d, same specimen as in c, after separating from fixed point; g–h, successive positions of flagellum shown in f; i–j, successive views of one specimen, in inseminated culture but not at egg; k–m, at surface of eggs (k, oscillating specimen).

Spermatozoa with acrosome filaments are also found near but entirely outside the jelly hull of the egg; in these no particular orientation with reference to the egg has been noted.

4. *Appearance of the unreacted spermatozoon.* Fresh sea water suspensions of sperm were spread thinly between slide and coverslip and individual spermatozoa examined as soon as they became sufficiently quiet. As seen in outline (Figs. 3, a–c and 5) the head is nearly circular. Closely applied to it is a narrow curved structure containing the middle piece and, apparently, a highly refringent circular body. The flagellum projects posteriorly as though from the middle piece but its actual point of origin is obscure. The flagellum is about 60 μ in length; its terminal

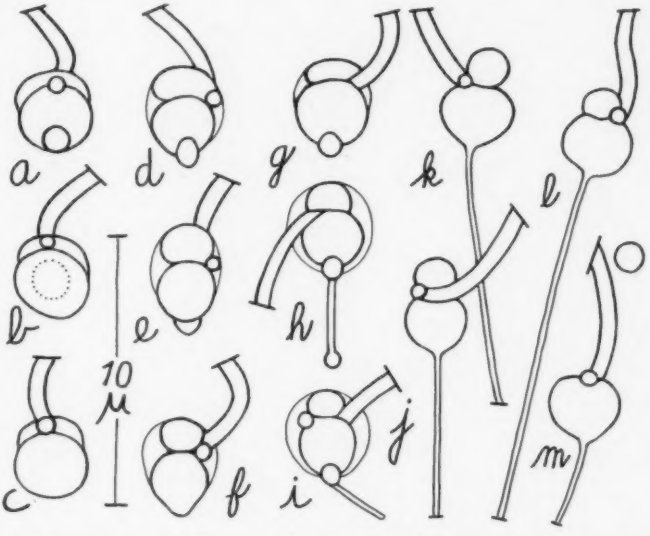
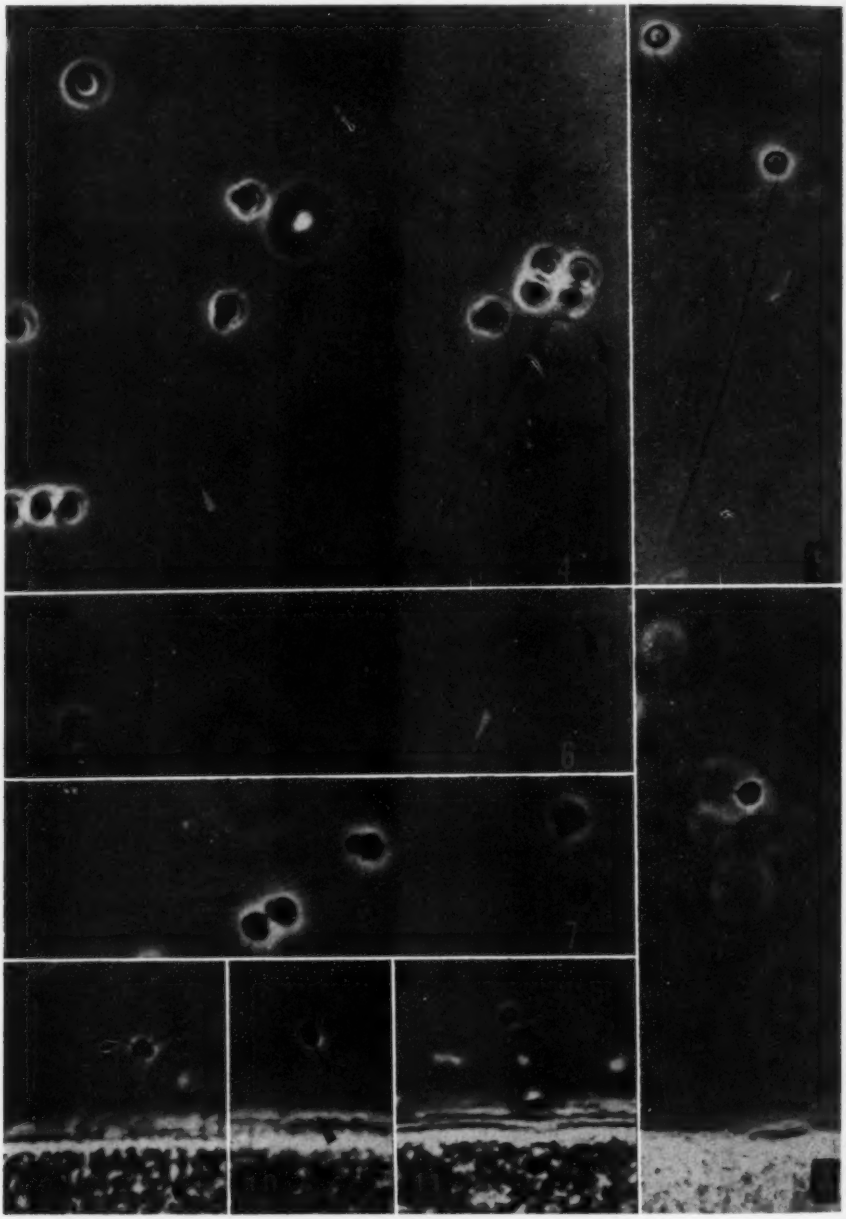


FIGURE 3. Spermatozoa of *Thyone briareus*, from sketches and photographs of living specimens. a–c, unreacted, in sea water; d–e, moribund, in aging sea water suspensions; f–g, partial reaction (?), in inseminated culture but not at egg; h–i, partial reaction (?), in alkaline sea water; j–l, reacted, as seen in all media described in text; m, reacted, entering egg, middle piece separated as seen occasionally.

portion or end piece is about 6 μ and much thinner than the main portion. The rounded acrosome, somewhat subterminal to the anterior part of the head, is often not seen, presumably because of the position of the specimen; it seems to intrude into the mass of the head (Fig. 3, a–c).

5. *Appearance of the reacted spermatozoon.* Reacted spermatozoa are morphologically indistinguishable from each other, whether they occur in egg water (Figs. 1, a and 4), in alkaline sea water (Figs. 1, c and 6–7), in association with the egg (Figs. 1, b and 8), or in untreated sperm suspensions; they are, however, markedly different from unreacted spermatozoa. The middle piece is round in outline, forming with the head the configuration of a figure eight (Figs. 1, a–c and 3, j–l). The flagellum emerges posterolaterally from between them, com-



FIGURES 4–11.

monly seeming to arise from the head (Figs. 1, a–c and 3, k–l). The refringent circular body lies near the point of contact between head and middle piece (Fig. 13). Anteriorly, from the acrosome region, projects the acrosome filament, frequently at an approximate right angle with the base of the emerging flagellum (Figs. 1, a–c and 5).

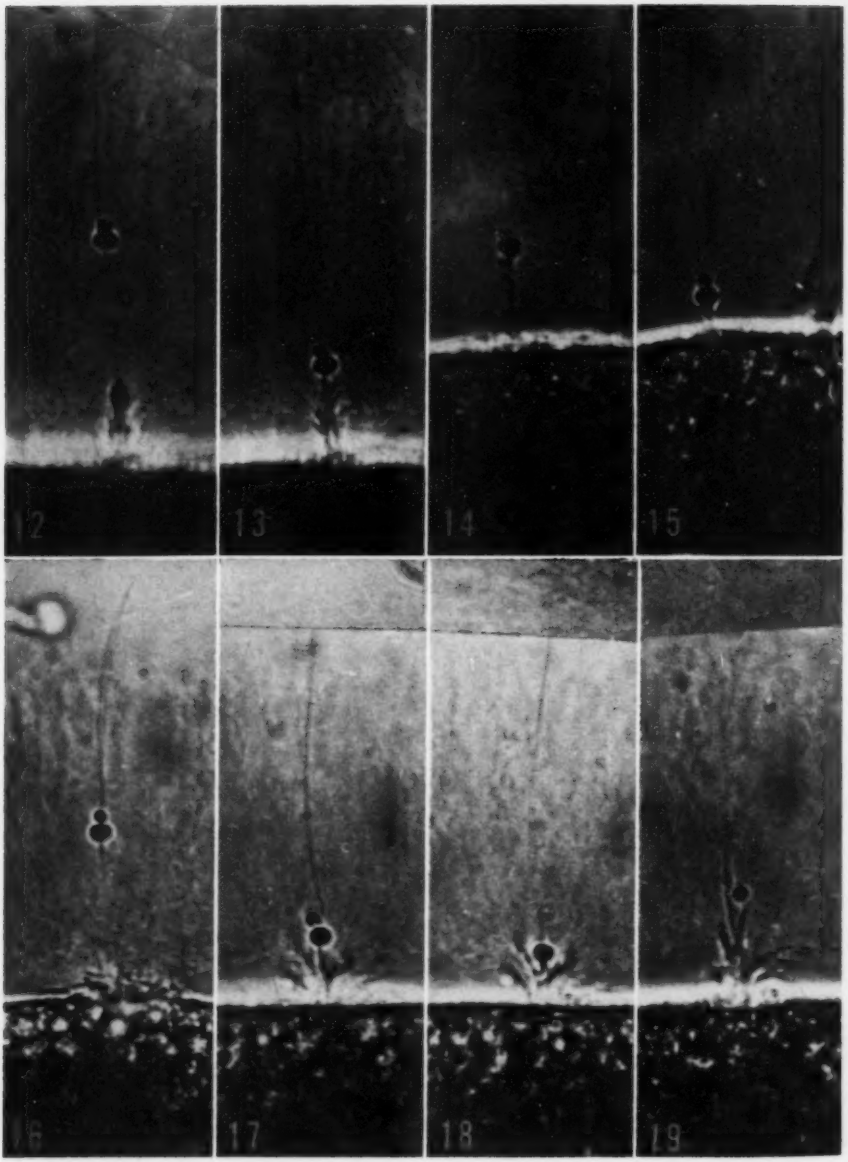
Successive stages of a specific reacting specimen have not been observed. Under certain conditions individuals are found like those shown in Figure 3, d–e (from aging sperm suspensions), Figure 3, f–g (in inseminated cultures) and Figure 3, h–i (in alkaline sea water). Here the enclosing membrane is loosened; the head, middle piece and refringent body are rounded and clearly discrete; and the flagellum emerges laterally, much as reported by Dan (1954a) for moribund spermatozoa of starfishes. The acrosome, however, is an additional balloon-like structure (*e.g.*, in Fig. 3, d) whose contents, under phase contrast, appear pale and diffuse as compared with the darker, seemingly more dense, head. Occasionally there is a short acrosome filament (Fig. 3, i) which is sometimes slightly thicker and ends in a rounded knob (Fig. 3, h). Though enclosing membranes are not prominent in fresh specimens, whether reacted or not (Figs. 3, a–c and j–l), it may be that these spermatozoa with loosened membranes hold clues to the intermediate stages of the acrosome reaction; except for the appearance of the membranes, Figures 3, g–i, for example, might be transitions from Figure 3, a to Figure 3, l.

6. *Dimensions of acrosome filament.* It has not been ascertained what length characterizes the average, fully reacted acrosome filament but most of those examined ranged between 35 μ and 65 μ. Several spermatozoa like the one shown in Figure 8 (which had attached but failed to enter the egg) had filaments measuring 46–48 μ. The acrosome filament of the spermatozoon shown in Figure 6 (and schematically in Fig. 1, c) measured 75 μ and exceeded the length of the flagellum. In one exceptional case the filament measured 90 μ. The acrosome filament is extremely tenuous, its diameter being of the general order of magnitude of that of the end piece of the flagellum.

B. *Asterias forbesii and Asterias vulgaris*

In both species reacted spermatozoa were found in fresh living preparations of inseminated eggs. Except that their acrosome filaments were very much shorter, the reacted spermatozoa of both species looked very much like those of *Thyone*. The heads were somewhat smaller but the flagella measured about 55–60 μ in length, including the short filamentous end piece. In *A. forbesii* the maximum length of the acrosome filament was approximately 25 μ; acrosome filaments attached to but failing to enter eggs (Figs. 1, e, 10 and 11) measured from 15–22 μ. The same order of magnitude prevailed in *A. vulgaris* (Fig. 2, m). In both species some reacted spermatozoa were found with their acrosome filaments directed *away* from the eggs (Figs. 1, d and 9).

FIGURES 4–11. Unretouched photographs of living spermatozoa; all to scale shown in Figure 4, representing 50 μ. Black arrows point to acrosome filament, white arrows to end piece of flagellum. Figures 4–8, *Thyone briareus;* Figures 9–11, *Asterias forbesii.* All figures show reacted spermatozoa with acrosome filament except Figure 5, which shows unreacted specimens. Figure 4, in egg water; Figures 6–7, in alkaline sea water; Figures 8, 10–11, at surfaces of eggs but did not subsequently enter; Figure 9, near egg but directed away from it.



FIGURES 12–19. Unretouched photographs of living spermatozoa penetrating egg of *Thyone briareus*. To same scale as in Figure 4. (All Figures except 15 and 16 have had non-relevant pieces spliced in to upper portions.) Figures 12–13, successive views of slender cone with broad

C. *General nature of the acrosome filament*

Some properties of the acrosome filament may now be described. As they are apparently much the same in both *Thyone* and *Asterias,* and whether they occur in egg water, alkaline sea water or in inseminated cultures, reference to species and medium will be omitted here but may be found with the appropriate illustration. Despite its thread-like dimensions the filament is usually straight (Figs. 2, i, 4, 6 and 11) or curved only in very wide arcs (Figs. 2, a and 8). Figure 2, k shows a specimen which was attached to (but did not enter) an egg; the filament curved slightly as the head oscillated with the motion of the flagellum. When not at the

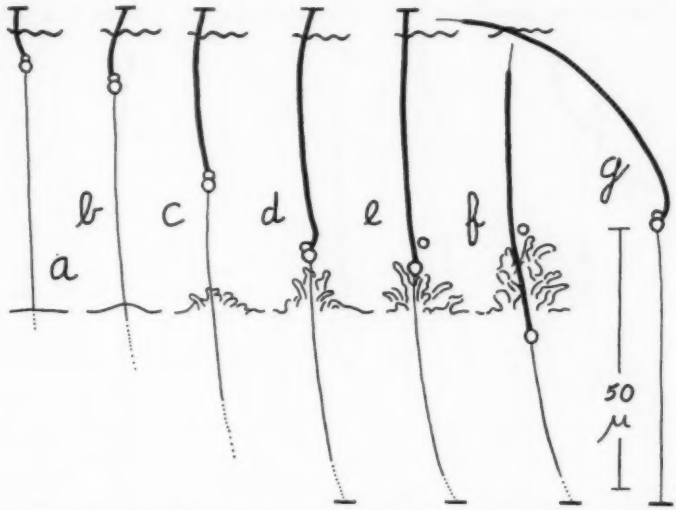


FIGURE 20. a–f, scheme of sperm entry process in *Thyone briareus;* part shown in dotted line represents minimum additional length of acrosome filament presumed but not actually seen to enter egg. g, reacted spermatozoon from alkaline sea water suspension for comparison of length of acrosome filament. a, presumed very early stage based on spermatozoon which failed subsequently to enter; b, low broad incipient cone rises as acrosome filament proceeds into egg proper; c–f, successive stages of sperm entry, acrosome filament enters egg proper as advance integral part of spermatozoon (middle piece, left outside in this case, frequently enters egg). In subsequent stages, entire flagellum passes into egg proper.

egg the distal portion and/or tip of the filament often sticks to the slide or coverslip; for example, the specimen shown in Figure 2, i–j, moved suddenly, causing the non-adherent proximal part of the filament to curve sharply. Other partly adhering, sharply curved filaments are shown in Figures 2, b and 2, e. The acrosome filament *may* even break. This could explain the spermatozoon shown in Figure 2,

base partly retreated; note acrosome filament in cone. Figures 14–15, successive views of one specimen, very slender cone; sperm head did not subsequently enter egg proper. Figures 16–19, successive views of specimen in which low broad cone gave rise to filose projections which continued growing outward even after sperm head had entered egg proper; no tall slender cone formed in this specimen; middle piece failed to enter.

c–d; when first seen it was oscillating like a pendulum with a short stem. Suddenly it began to whirl freely in many directions, and its filament seemed even shorter; the presumed adhering distal part of the filament was not observed. A number of spermatozoa have been seen with the filament bent permanently (Figs. 2, f, l, m, 7 and 10).

II. Sperm entry

A. *Thyone briareus*

Many individual eggs were examined, both from natural sheddings and stripped from the gonads. As lightly inseminated cultures of the naturally shed eggs gave 98–99% cleavage and more than 90% active larvae, it was evident that these were

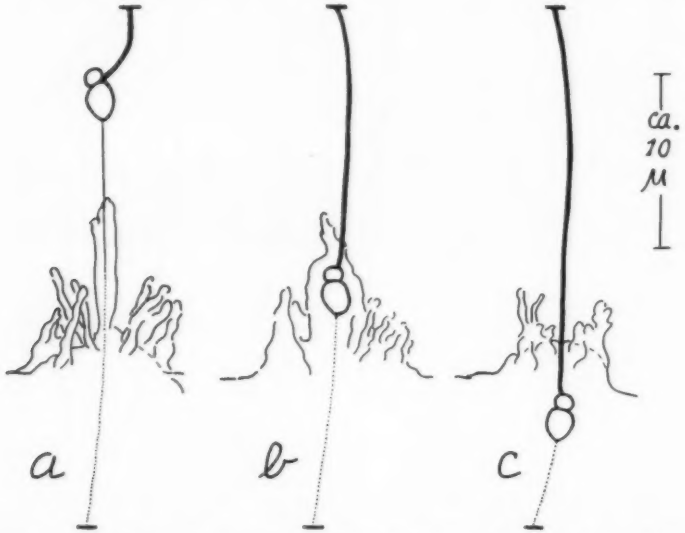


FIGURE 21. Successive stages of sperm entry in *Thyone briareus*, from sketches of a living specimen. A broad cone with filose projections elevated one moderately slender sleeve about the inmoving acrosome filament. Acrosome filament seen in outer part of cone: solid line. Presumed further course of filament: dotted line.

normal eggs. On the other hand, cleavage was never observed in inseminated cultures of the stripped eggs. Yet, whichever the source, a number of spermatozoa entered every egg which was observed individually in slide and coverslip preparations. Generally, the entry was more rapid and the height of the cone less great in the naturally shed eggs.

A scheme of the entry process is shown in Figure 20. The spermatozoon does not swim through the thick jelly hull; its first contact with the egg surface is made by means of its acrosome filament. Presumably a very early stage would appear as in Figure 20, a. At the earliest stage noted frequently in successful entry, the main body of the spermatozoon had already progressed part way through the jelly and its acrosome filament was already associated with the broad low hyaline in-

cipient cone (Fig. 20, b). In some instances, as the spermatozoon continued moving inward, filose projections arose from the cone and continued growing outward even after the sperm head had passed through the cone into the egg proper (Figs. 16–19).

Some common variations in the cone are shown as they appeared in specific living eggs. The cone shown in Figures 16–19 never rose extensively above the egg surface. Sometimes a moderately slender projection would move outward like a sleeve ascending the inmoving acrosome filament, and then retract after the sperm head had entered the cone, as in Figure 21, a–c. In this specimen the cone remained broad at the base throughout the entry process but often an initially

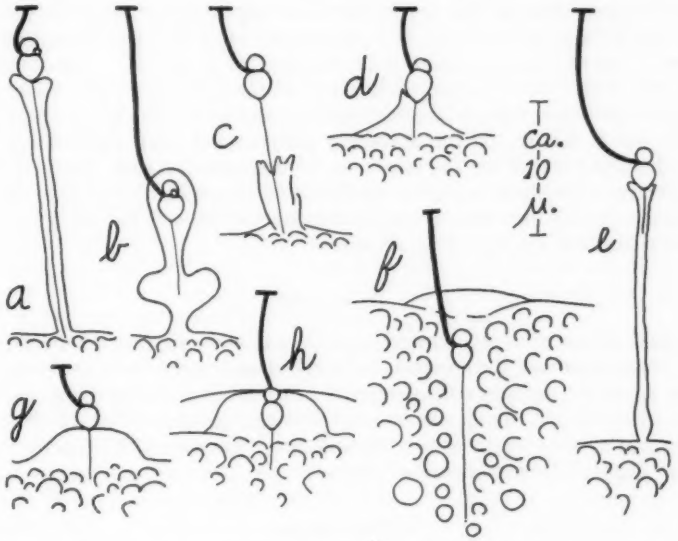


FIGURE 22. Variations in sperm entry, from sketches of living specimens. a–f, *Thyone briareus;* g–h, *Asterias forbesii.* Acrosome filaments shown only to depth actually seen. a–b and c–d, successive views of two specimens, respectively; e, very slender cone embracing acrosome filament; f, acrosome filament within egg proper; g–h, from two specimens of *A. forbesii,* successive stages which preceded stage closely resembling that shown in f.

broad base would retreat partly or wholly so that the only obvious external cone would be a single slender projection (Figs. 12–13, 14 and 22, a, c and e). Transitory changing dilations appeared along such cones (Figs. 12–13 and 14). The cones were sometimes so slender that they might easily have been called "filaments" by an observer not cognizant of the existence of the much more delicate acrosome filaments (Figs. 14, 22, a and e). There was also variation in the distance from the egg proper at which the sperm head entered the cone (*cf.* Figs. 22, b and d). In the specimen shown in Figure 22, c–d a column of hyaline protoplasm continued to grow outward for some time after the sperm head had passed into the egg proper.

Although the middle piece ordinarily entered the egg with the sperm head (Figs. 21, 22, b and f) it sometimes became separated (Fig. 3, m) and remained outside

altogether (Fig. 19). Occasionally an apparently detached middle piece eventually passed into the egg. Several times a detached middle piece was seen to vibrate vigorously with the movement of the flagellum while the head continued moving on into the egg without pronounced vibration.

The entire flagellum passes into the egg. However, it was sometimes not seen or seen only with difficulty within the cone, even in cases in which it was observed continuously until finally the end piece disappeared into the cone.

The much more tenuous acrosome filament was also seen only with difficulty after it had been enveloped by the cone (Figs. 12–14 and 22, c–d). Sometimes only a portion of the filament could be seen (Fig. 22, e). In other cases a part of the filament would be seen and then disappear with the changing condition of the cone (Figs. 22, a–b). Within the egg proper, observation was further hindered by the presence of yolk granules (Fig. 15) and it was only in some specimens that the acrosome filament was seen there with certainty (Fig. 22, f). Occasionally some disturbance of the yolk granules in advance of the entering sperm head suggested the presence of an acrosome filament not actually seen. In the scheme of sperm entry shown in Figure 20 the acrosome filament has been represented by solid lines to the depth within the egg that it has sometimes been seen, and in dotted lines to the depth to which it might be presumed to penetrate, judging by the known lengths of acrosome filaments as seen in alkaline sea water (Fig. 20, g) or attached to but not entering the egg (Fig. 20, a).

B. *Asterias*

A small number of polyspermic eggs of *A. forbesii* were examined. In the earliest stages observed the spermatozoa were already quite close to the egg surface. In some cases the acrosome filament was seen within the cone (Fig. 22, g); in a few it extended into the protoplasm of the egg proper (Fig. 22, h). Several times an appreciable length of the filament was seen within the egg, very much as shown in Figure 22, f representing *Thyone*.

Discussion

A. Acrosome filaments

The acrosome reaction resulting in the production of a filamentous structure, the acrosome filament, first demonstrated by Dan, has been shown here to occur also in the holothurian, *Thyone briareus*. Whereas the longest previously reported acrosome filament, that of starfishes (Dan, 1954a), measured 22–28 μ, the acrosome filament of *Thyone* may reach two to three times this length and can even exceed the length of the flagellum.

Recently Rothschild and Tyler (1955) have questioned that the acrosome filament is formed by means of an acrosome reaction, in the sense of Dan. Their reservations are based on observations of untreated sperm suspensions of two species of mollusc and two species of sea urchin, viewed by both phase contrast and electron microscopy. They state that in such preparations generally two types of spermatozoa are found, one type with a short "acrosomal filament" and one with a long "acrosomal filament." Two such types of sperm are shown, for example, for the sea urchin *Echinocardium cordatum* in their Figures 8 and 9. Their Figure 8

shows a spermatozoon with a fairly long and knobbed acrosome, and it appears as previously described for this species by Vasseur (1947) and by Afzelius (1955); it was the form invariably found in a living sperm suspension. Their Figure 9, however, shows a spermatozoon with a profoundly different appearance; a filament much longer than that of the other type extends from the apex of the head, the middle piece is excentric, and the flagellum and filament lie nearly at right angles to each other. In all these features the spermatozoon resembles the reacted spermatozoon of the sea urchin as described by Dan (1952). Furthermore, Tyler (1952) has previously reported that fertilizin-treated sea urchin spermatozoa show just such a displacement of the middle piece from the normal position to a postero-lateral one. There seems little doubt, then, that the spermatozoon shown by Rothschild and Tyler in their Figure 9 represents a reacted spermatozoon and the long filament extending from the apex of the head is an acrosome filament, in the sense of Dan.

Perhaps the reservations of Rothschild and Tyler might have been obviated by the presence of quantitative data in the reports by Dan; and similarly it would be very interesting if Rothschild and Tyler had presented quantitative data of *treated* sperm suspensions to compare with their results of the untreated suspensions. Any reservation regarding the existence of the phenomenon of the acrosome reaction should be dissipated by the quantitative data presented here for *Thyone,* in which it is clearly demonstrated that the treated preparations show significantly greater percentages of reacted spermatozoa with acrosome filaments than untreated preparations, *as observed exclusively in living material.* The tremendously long acrosome filaments of *Thyone* make these observations relatively easy.

The occurrence of the two types of spermatozoa observed by Rothschild and Tyler in their untreated preparations may have been caused by "contact" or other as yet unknown factors which supervene during preparation of the sperm suspensions. It is important to note that in *Echinocardium cordatum* Rothschild and Tyler found the long "acrosomal filaments" only in *fixed* material; the possibility exists that in this case the fixative, or some feature of the process en route to fixation, may have been the stimulus for the acrosome reaction. In this connection an observation of Lillie (1912) on *Nereis* now becomes extremely interesting; he noted that in the *living* spermatozoon the perforatorium (acrosome) was shaped like the spike of a helmet and was *shorter* than in the *fixed* spermatozoon, whether the latter was free or attached to an egg; in this fixed material the acrosome had the shape of a filament. What Lillie observed in the fixed material would seem to be an acrosome filament resulting from an acrosome reaction (possibly caused by fixation). Recently Metz and Morrill (1955) have also studied fixed spermatozoa of *Nereis;* the acrosome filament was present in low percentage in normal sea water controls, and in significantly higher percentage in spermatozoa which had been treated with fertilizin. A more detailed discussion of this and related matters is presented elsewhere (Colwin and Colwin, 1956).

B. Sperm entry

In *Holothuria atra* (Colwin and Colwin, 1955a) the acrosome filament behaves as an integral part of the sperm head, enters the egg intact and remains not appreciably altered until at least well within the egg proper. A relatively broad cone rises to surround the entering filament like a sleeve. Frequently a short narrow

projection creeps up the acrosome filament in advance of the main body of the cone. In *Thyone,* although the cones are narrower, and the acrosome filaments a great deal longer, the major aspects of sperm entry are much the same, namely, the acrosome filament enters the egg as an integral part of the spermatozoon and remains essentially intact at least during its initial entry into the egg proper; the substance of the cone moves outward enclosing *the acrosome filament which has already established the first true contact between spermatozoon and egg proper.* But whereas in *H. atra* all the observations were made on eggs which had been stripped from the gonads artificially, in *Thyone* not only stripped eggs but also normally shed eggs, controls of which developed normally, showed essentially the same picture. This strengthens the suggestion, made previously, that sperm entry as described for *H. atra* reflects the essentials of the entry phenomenon in the normal egg of that species, particularly with respect to the behavior of the acrosome filament.

The tall slender type of cone usually seen in *Thyone* strongly suggests the reception cone described by Hörstadius in *Holothuria poli* (1939a) and the starfish *Astropecten aranciacus* (1939b). In *Thyone,* however, since the cones can be seen to embrace the already established inmoving acrosome filament, there is no basis for their interpretation as the agents of first contact between egg and spermatozoon, as has sometimes been held. A more extensive discussion of the mechanism of sperm entry and a reinterpretation of the events of entry as described in echinoderms by earlier workers will be found elsewhere (Colwin and Colwin, 1955a and 1956).

From the measurements in the present observations it is evident that the acrosome reaction in *Thyone* is capable of producing acrosome filaments much longer than the depth of the jelly hull of the egg. The question arises: how long must the acrosome filament be in order to initiate successful sperm entry? As the spermatozoon does not swim through the jelly in *Thyone,* it seems that the minimum length necessary would be that of the thickness of the jelly.

The authors have never witnessed a subsequently entering spermatozoon at the exact moment of its first contact with the egg in *Thyone,* or indeed in *H. atra* and the three species of *Asterias* which have been studied. In all of these it has been considered likely that the earliest stage would resemble that seen when spermatozoa attach to the egg but fail subsequently to enter, as in Figures 8, 11 and 20, a. However, since it is known that the acrosome filament of *Thyone* can be longer than in such attached spermatozoa, the possibility certainly exists that these attached specimens represent a stage later than the earliest one. Inasmuch as the filament of successfully entering spermatozoa moves into the egg, perhaps in these attached non-entering spermatozoa some distal portion of the filament may already extend into the egg.

Summary

1. As shown by quantitative data, spermatozoa of *Thyone briareus* treated with egg water or alkaline (ammoniated) sea water undergo an acrosome reaction resulting in the production of acrosome filaments in a percentage significantly greater than found in untreated controls. Acrosome filaments are also found in inseminated cultures of eggs, either associated or unassociated with the eggs.

2. The acrosome filaments of *Thyone briareus* are exceptionally long and may even exceed the thickness of the jelly hull (55 μ) or even the length of the flagellum (60 μ).

3. Reacted spermatozoa of *Asterias forbesii* and *A. vulgaris* seen in inseminated cultures of eggs have acrosome filaments measuring about 15–22 μ.

4. The general nature of the acrosome filament is much the same in both species of *Asterias* and in *Thyone,* regardless of the stimulating agent. Though thread-like in dimensions it is usually straight, or curved only in a wide arc. However, it is capable of curving sharply, bending and perhaps even breaking. The tip or distal portion often sticks to the glass of the slide.

5. At sperm entry in *Thyone* and *Asterias* the acrosome filament makes the initial contact with the egg and then enters the egg as the first element of the spermatozoon of which it is an integral part. In *Thyone* the cone is sometimes so slender as to appear filamentous itself, even though it contains the acrosome filament.

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POLYPHENOL OXIDASE ACTIVITY AND PIGMENTATION IN SNAIL TISSUES [1]

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Relatively little has been done to elucidate the mechanisms of intermediary metabolism in molluscs despite their recognized medical and economic importance (von Brand, 1952). A knowledge of such mechanisms would seem to be an essential preliminary to any rational investigation of these forms as parasite vectors or any logical attempt toward their control. In addition, comparative information concerning metabolically important reactions in different animals is a valuable aid to our understanding of the patterns of animal metabolism in general.

As part of an over-all study of snail physiology and biochemistry various aspects of the functioning of the midgut gland (hepatopancreas) of certain fresh water snails have been investigated. During one phase of this program a study was made of the mechanisms thought to be responsible for the presence of the reasonably large amount of pigment normally found in this gland in the fresh water operculate snail, *Ceratoides cornvarietis* (Linné, 1758). The deposition of melanin pigments in various tissues is a characteristic activity in many organisms, but the function, if any, of such pigments is frequently obscure. Since the pigmentation is the normal result of metabolism in the liver of the snail and since it has been suggested that the oxidative components of the series of chemical transformations resulting in melanin formation may be linked to the electron transfer systems of certain aerobic dehydrogenases (Baldwin, 1952) it was felt that a study of the function of snail liver must include some references to these reactions. The present report deals with some of the characteristics of the polyphenol oxidases in snail liver and their probable connection with melanin formation.

Methods

The fresh water snail, *Ceratoides cornvarietis,* used in the present study was initially obtained from a stream near Rio Piedras, Puerto Rico. The animals used in the present study were raised in this laboratory from the original stock. This snail is not a vector for any known medically important parasite, but was chosen for the initial studies in snail physiology because of its large size, abundance, hardiness in the laboratory, and, importantly, because of the fact that in this snail, by careful dissection, the gonads could be fairly well separated from the midgut gland. It was expected that the results obtained and techniques developed in the study of this snail would allow a more effective approach to the later study of more medically

[1] This study was supported (in part) by a research contract with the Office of the Surgeon General, U. S. Army and (in part) by a grant, No. E-374, from the National Institutes of Health, Public Health Service.

important forms. The snails were kept in the laboratory in constantly aerated pond and well water aquaria. They were not starved (Baldwin, 1938) before experimental use as no significant improvement in the consistency of results was obtained in this manner.

The assays for enzyme activity were carried out on cell-free homogenates (Potter and Elvehjem, 1936) prepared in cold, glass-distilled water from tissue samples carefully dissected from large mature snails. The use of varying salinities as suspending media failed to significantly alter the results. In the preliminary experiments the tissues were segregated according to the sex of the animal, but when no fundamental differences were observed this procedure was abandoned. The homogenates were uniformly centrifuged at low speed ($600 \times g$) to remove the major part of the heavy inert pigment before being diluted to a final 5% concentration. This dilution effectively prevented any oxygen consumption by endogenous metabolism. All of the foregoing procedures were carried out at 4° C. and the reaction flasks were kept thoroughly chilled until placed in the water bath. The reaction mixtures were assayed in Warburg flasks in an atmosphere of air at 25° C.

TABLE I

Per cent dry weight, total nitrogen, and the ether-soluble fraction of snail liver

% dry wt.		N_2 mg./gm. dry wt.		Lipid mg./gm. dry wt.	
Male	Female	Male	Female	Male	Female
27.4	25.6	74.8	68.2	164	230
27.0	28.9	75.2	67.4	144	209
27.0	27.1	72.9	68.2	151	214
28.1	28.2	73.4	69.1	153	198
27.2	26.8	72.8	66.9	167	207
27.0	26.9	72.9	67.0	172	222
26.9	28.1	74.3	68.2	154	214

Suitable controls were included in each run. Initial runs were made to insure optimum conditions in regard to enzyme concentration, substrate concentration, and pH. Dry weight determinations were made to check against differences in individual tissue samples as were total nitrogen determinations as a crude test for total protein (micro-Kjeldahl) and fat determinations (ether-soluble weight loss method).

RESULTS AND DISCUSSION

During the course of the assays for specific enzyme activity periodic checks gave the results on dry weight, total nitrogen, and the ether-soluble fraction which are summarized in Table I. Although both male and female livers have *ca.* 27% dry material, the male liver contained somewhat more nitrogen and less ether-soluble material. In dissecting out the female liver some difficulty was encountered in completely freeing the liver from extraneous tissues. The differences in the nitrogen and fat data between the sexes, therefore, are possibly a reflection of the difference in the closeness of the anatomical relationship of the liver and gonad. Apparently, however, this difference in the tissue samples was not significant from

TABLE II

Influence of enzyme concentration, substrate concentration (catechol), and pH on the activity of polyphenol oxidase activity in snail liver homogenates. All but the experimental substances were at optimum concentration

Enzyme concentration (mgm. snail liver, wet wt.)			5	10	25	30
Q_{O_2}			26 (23–29)	56 (54–58)	108 (99–115)	111 (101–121)
Substrate concentration (catechol in micromols)			10	20	50	60
Q_{O_2}			25 (23–29)	42 (38–47)	99 (95–110)	103 (98–108)
pH	6.8	7.2	7.6	8.0	8.4	8.8
Q_{O_2}	41 (35–52)	73 (67–79)	92 (86–99)	106 (97–111)	91 (87–96)	62 (53–71)

the standpoint of the enzyme activity, since no variation between sexes in this regard was obtained from the assays.

In Table II are given the results of studies on the influence of enzyme and substrate concentration and the pH on the activity of the snail liver polyphenol (catechol) oxidase activity. Optimum conditions were obtained with a reaction mixture containing 25 mgm., wet weight, of cell-free homogenate, 50 micromols of substrate (catechol), and 0.033 *M*, final, potassium phosphate buffer at a pH of 8.0. The addition of neither methylene blue, diphosphopyridine dinucleotide, nor cytochrome *c* to the reaction mixture to serve as a hydrogen carrier was of any benefit to this system. From this it would seem that the system is an aerobic oxidase rather than an aerobic dehydrogenase. The replacement of the air in the reaction system with 100% oxygen was also of no significance in promoting higher activity. The enzyme was found to exhibit a marked stability for several hours at room temperature and it was still highly active after refrigeration for 96 hours.

TABLE III

Activity of the monohydric and polyhydric phenol oxidases in snail liver homogenates. Reaction flask contained 25 mgm. (wet weight) liver homogenate, 0.1 M pH 8.0 phosphate buffer, 50 micro. mol. substrate, 1 ml. 20% NaOH in center well. Each figure is the average of closely approximated duplicates

Substrate	Q_{O_2}					
	No. 1	No. 2	No. 3	No. 4	No. 5	No. 6
p-Cresol	0	0	0	0	0	0
Phenol	16	0	0	0	0	0
Tyrosine	0	0	9	0	0	0
Guaiacal	6	0	21	0	0	0
Catechol	104	107	107	106	110	99
Pyrogallol	142	141	144	145	132	134
Adrenaline	72	79	75	69	72	73

In the frozen or lyophilized state it was stable up to six months. In this regard the enzyme resembles the tyrosinase of earlier workers (Lardy, 1949).

However, the enzyme system in snail liver was observed to seemingly possess a generic specificity for polyhydric phenols (Table III). Negligible activity was

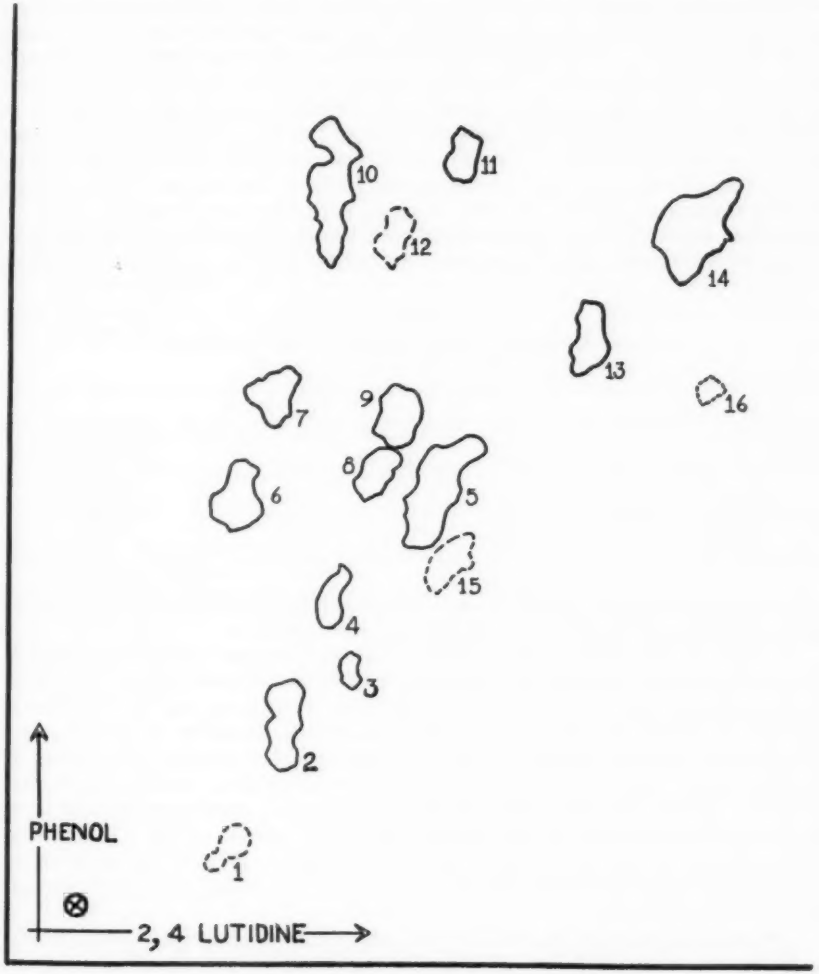

FIGURE 1. Two-dimensional composite chromatogram of the liver from *C. cornvarietis* extracted for free amino acids. Broken lines indicate relatively low concentration. 1—aspartic acid; 2—glutamic acid; 3—serine; 4—glycine; 5—alanine; 6—lysine; 7—arginine; 8—citrulline and/or glutamine; 9—β-alanine; 10—unidentified polypeptid; 11—proline; 12—unidentified; 13—valine; 14—methionine and leucine; 15—threonine; 16—unidentified trace.

occasionally obtained on p-cresol, phenol, tyrosine, or guaiacol substrates whereas catechol, adrenaline, and, to an even greater extent, pyrogallol provided relatively excellent substrates for the system. From these data it appears that the enzyme which oxidizes the polyhydric phenols in snail liver is not identical with that reported for certain plant tissues (Nelson and Dawson, 1944). The enzyme described by these earlier workers (tyrosinase) was active against both polyhydric phenols and monohydric phenols, although the activity against the latter was usually somewhat unstable. It would seem that the enzyme described herein more closely approximates the catecholase studied by Graubard (1939) which also attacks only polyphenols. However, in this regard it should be remarked that the activity of tyrosinase against catechol has been reported as being *ca.* 124 times that against tyrosine (Kubowitz, 1938). Therefore, it was considered possible that the amount of tissue used in the major part of the present study was insufficient to demonstrate the action against tyrosine and other monophenols, especially since the maximum polyphenol oxidase activity measured was considerably less than that demonstrable on potato and mushroom preparations. In testing this, however, no combination

TABLE IV

Survey of polyphenol oxidase (catecholase) activity in snail tissues. Reaction mixture as in Table III

Tissue	Q_{O_2}		
	No. 1	No. 2	No. 3
Liver	105	108	107
Foot	29	22	20
Mantle	68	64	55

of reactants was found which at any time permitted anything but periodic and highly insignificant activity with monophenol substrates.

In view of the demonstrated inactivity of snail tissues against monophenols, liver extracts were qualitatively analyzed for free amino acids, using two-dimensional ascending paper partition chromatography with phenol and 2, 4-lutidine as solvents, to determine the presence and metabolic availability of tyrosine as a substrate for enzyme activity *in vivo*. Glutamic acid, aspartic acid, valine, β-alanine, serine, glycine, arginine, lysine, alanine, proline, leucine, methionine, citrulline and/or glutamine were readily identifiable in good quantity but at no time was there detected any appreciable amount of tyrosine (Fig. 1). This apparent absence of tyrosine is consistent with the failure of the tissue preparations to oxidize monohydric phenols and the failure of the intact snail to produce pigment from tyrosine substrates.

Although maintaining the snails in the presence of catechol for 7–14 days prior to the enzyme assays failed to elicit any increased activity, it was observed that a decline in enzyme activity accompanied the decolorization of the tissues of snails kept for several weeks in tap water without food. This observation seems to associate the enzyme action with the pigmentation of the snail *in vivo*, particularly since other metabolic systems not concerned with melanin production (respiratory

enzymes, transaminase, and the amino acid oxidases) were not similarly affected (current unpublished results).

Further evidence of an association of the polyphenol oxidase activity with pigmentation was given as the result of enzyme studies on other tissues of the snail (Table IV). Both the mantle and the foot, in order, showed some activity but far less than that described above for the liver. It was interesting to note that the level of activity in the three tissues corresponded roughly to the degree of pigmentation, the foot in this snail being relatively free of coloration.

Acknowledgment is made to Dr. Harold Harry, U. S. Army Tropical Research Medical Laboratory, San Juan, Puerto Rico, for the initial collecting of the snails used in this study.

Summary

A study was made on the polyphenol oxidase activity of snail tissues. The liver showed higher activity than either the mantle or foot; however, even in this organ only polyhydric phenols were effectively oxidized. No consistent activity on monophenol substrates was found. The possible association of the polyphenol oxidase system to the heavy pigmentations in the liver was discussed.

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AN ANALYSIS OF CELL GROWTH AND METABOLISM IN THE CRAYFISH (PROCAMBARUS ALLENI)

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In a recent paper I proposed a general scheme relating cell growth to organism growth with special reference to the problem of body size and metabolic rate (Davison, 1955). Three hypothetical cases were considered. (1) If growth of the animal is exclusively by cell enlargement, cell surface and external body surface each vary directly as the body weight raised to the 2/3 power. (2) If growth is characterized by the progressive formation of cells of fixed size, cell surface will be directly proportional to the body weight raised to the one power. (3) Growth involving the simultaneous addition and enlargement of cells results in cell surface varying as the body weight raised to some power intermediate between 2/3 and 1. In the 1955 paper it was demonstrated that post-metamorphosis growth of frog skeletal muscle is strictly by enlargement of fibers present at the completion of metamorphosis, no new fibers being formed. Both metabolic rate (cc. O_2/hr./gm.) and muscle surface concentration were directly proportional to the body weight raised to the $-1/3$ power.[1] Thus the metabolic rate of the whole animal was directly proportional to the surface concentration of the skeletal muscle cells.

In the frogs studied, the geometric weight increases were small (10- to 50-fold). In further pursuing the problem, I thought it might prove interesting to examine an animal which undergoes a large geometric increase body weight. The crayfish (*Procambarus alleni*) weighs about 4 mg. at the time it becomes independent of the parent crayfish, and increases to an average adult weight of about 16 gm., a 4000-fold increase in body weight. If such growth were isogonic, and the result of cell enlargement, cells would undergo a 13-fold increase in linear dimension ($4000^{\frac{1}{3}}$). Similarly, if the surface law of metabolism were in effect, metabolic rate would undergo a 13-fold decrease over this weight range. Since such dramatic changes in metabolism and cell dimensions seemed unlikely, the crayfish was selected as an interesting object for investigation.

Materials and Methods

Animals

Here in the zoology department a breeding population of *P. alleni* has maintained itself in a concrete pool for about 5 years. The pool has a surface area of

[1] The usual form of the surface law equation is:

$$\text{cc. } O_2/\text{hr.} = k\ W^{\frac{2}{3}}$$

It is more convenient to express metabolism in the form of intensity, thus making it comparable with other information obtained in the form of concentration. Dividing both sides by grams:

$$\text{cc. } O_2/\text{hr./gm.} = k\ W^{-\frac{1}{3}}$$

Similarly, cm.^2 surface $= k\ W^{\frac{2}{3}}$ becomes:

$$\text{cm.}^2 \text{ surface/cm.}^3 \text{ (gms.)} = k\ W^{-\frac{1}{3}}$$

about 3 m.^{2} and is about 10 cm. in depth. Weak daylight is augmented by continuous illumination from a 300-watt bulb. In addition to the algae which grow in the pool, food is provided in the form of occasional frog carcasses and pellets of mouse food. The population usually consists of 10 to 15 adult animals with a variable number of young depending on the breeding history of the pool. The population can be assumed to be genetically quite homogeneous, since often the surviving adults are siblings from a single successful breeding.

Determination of respiratory rate

P. alleni is of the chimney-building variety and is able to survive under moist terrestrial circumstances. Adult animals frequently leave the pond and migrate about the laboratory, especially during rainy weather. It is thus possible to determine the respiratory rate in air with simple volumetric instruments similar to that employed by Flemister and Flemister (1951). These consist simply of two chambers separated by a manometer, one vessel containing the animal and CO_2 absorbent, and the other serving as a compensating vessel. The total volume of the animal chamber can be varied by means of a calibrated syringe, and the gas consumed is measured as the decrease in volume of the animal chamber required to maintain equal pressure in the two vessels. Three volumetric instruments were used. The first two, for larger animals, utilized animal chamber volumes of *ca.* 500 and 50 cc. and oxygen uptakes were measured with 10- and 1-cc. syringes, respectively. The third instrument consisted of two 5-cc. vials connected by a manometer. The volume of the animal chamber was varied by means of a mercury column in a 0.2-cc. calibrated pipette, driven by an ordinary screw clamp. For the smallest animals (4 to 35 mg.) simple capillary respirometers were employed, consisting of vertically mounted capillaries (*ca.* 0.75 mm. bore) attached to horizontal vessels by means of No. 00 rubber stoppers. Vessel volumes were about 0.2 cc. for the smaller and 0.5 cc. for the larger animals. Four instruments, closely matched for final gas volumes and capillary bores, were used in a determination, three being experimental vessels with the fourth serving as a control and thermobarometer. A three-mm. column of kerosene was used as the indicator drop. Results obtained with the capillary instruments agreed well with those obtained with the volumetric instruments on animals of comparable weight. All instruments were carefully calibrated with mercury.

Filter paper, saturated with 10% KOH, was used as CO_2 absorbent, with plastic screening protecting the animal from injury. Experiments in which pure oxygen was flushed through the instrument did not differ significantly from experiments in air. Accordingly, the experiments reported here were performed in atmospheric air. Heistand (1931) reports a T_e of about 40 mm. Hg for oxygen consumption in *Cambarus,* a value well below that approached in the present experiments. The water bath was maintained at 25.1 ± 0.05 C.°

Twenty-four to 48 hours prior to a determination, the animals were isolated in finger bowls or glass aquaria and maintained without food in order to minimize digestive and absorptive activity. After introduction into the respirometer, oxygen consumption was followed with the animal at rest until uptake became linear with respect to time. After the preliminary equilibration of 15 to 30 minutes, most determinations lasted from 60 to 180 minutes. Only one animal was used per

respirometer. The animals were blotted with filter paper and weighed fresh following the determination. No sex differences were observed. Oxygen consumption is expressed as cc. O_2/hr./gm. fresh weight, and gas volumes are reduced to standard temperature and pressure (760 mm. Hg and 0° C.).

No seasonal influence on breeding is apparent in the population, and the metabolism measurements reported here were obtained between September and March.

Determination of cell surface

The animal was weighed, decapitated and the abdomen removed. A small portion of abdominal flexor muscle was dissected out and teased apart on a glass

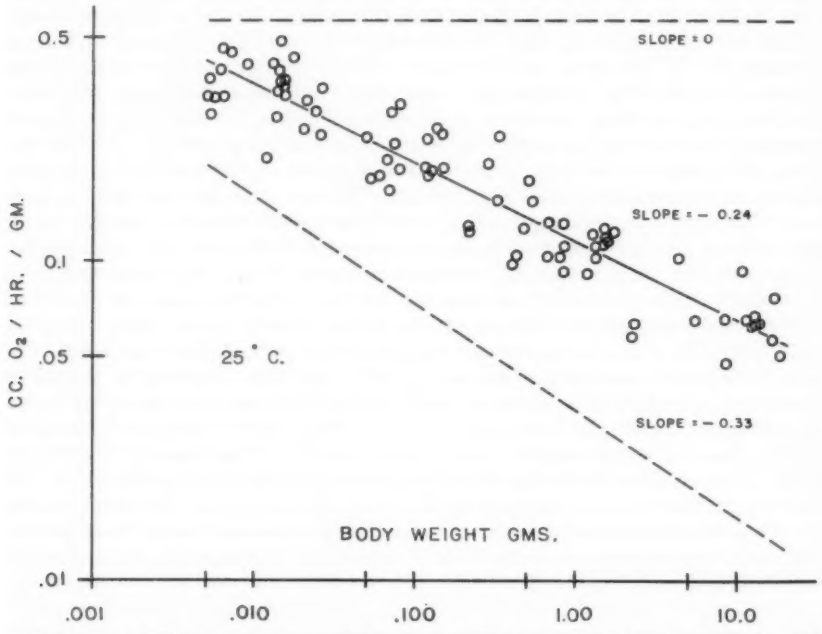

FIGURE 1. Resting oxygen consumption in *Procambarus alleni* plotted against the body weight in grams. Both ordinate and abscissa are in log form. The equation of the experimentally determined line is: cc. O_2/hr./gm. $= 0.115\ (W)^{-0.24}$. 25° C.

slide. The teased muscle was then stained with 0.75% acetocarmine in 45% acetic acid. It is necessary to stain the muscle cell nuclei in order to easily identify single fibers. The preparation was observed under a suitable power of the compound microscope. About 20 fibers were chosen at random, and the fiber diameters projected on paper by means of the camera lucida. A known linear dimension was projected from a stage micrometer, and the mean fiber diameter determined. For a sample of 20 fibers, the standard deviation was found to be about 20% of the mean. Assuming the fibers approximate long cylinders without free ends, their

surface is given by $2\pi rl$ and their volume by $\pi r^2 l$, so surface per unit volume is the former over the latter or $2/r$ $(4/d)$. This quantity, expressed as cm.^{2} surface/cm.^{3} fibers, is defined as the surface concentration of the muscle. Examination of fiber cross-sections indicates extra-fibral spaces less than 5% of the total area of the section.

Other measurements

Measurements were also made of abdomen length and breadth, eye diameter, and ommatidia facet size. The decapod ommatidium is a receptor unit of a definite

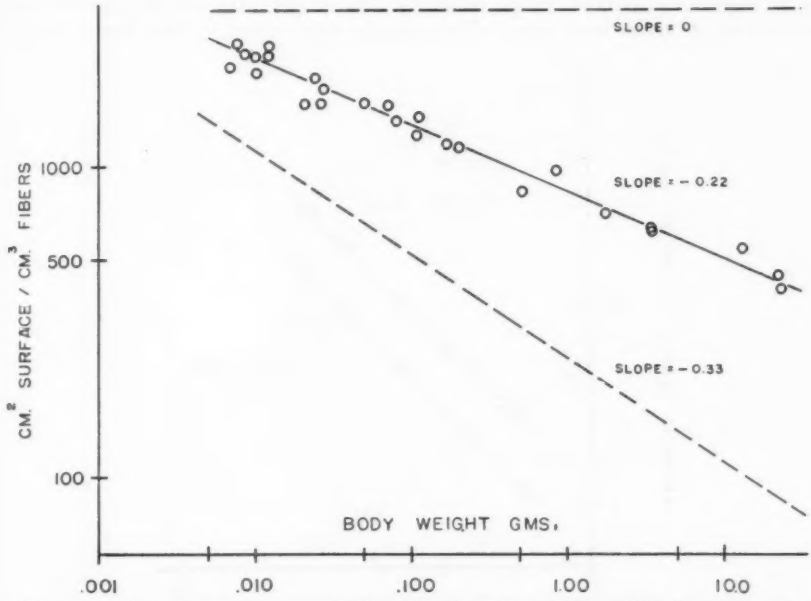

FIGURE 2. Muscle surface concentration plotted against body weight in grams. Both ordinate and abscissa are in log form. The equation of the experimentally determined line is: cm.^{2} surface/cm.^{3} fibers $= 830\ (W)^{-0.22}$.

number of cells (Schneider, 1902), so that an analysis of eye size and facet size allows an indirect examination of cell growth and organ growth using the familiar allometric equation of Huxley (1932). Eye diameter was obtained by observing the animal in dorsal aspect and taking [2] the diameter parallel to the longitudinal axis of the animal. Abdomen length was taken as the dorsal distance between the posterior margin of the cephalothorax and the base of the telson. Abdomen breadth is the ventral distance between the pleura of the first abdominal segment.

The size of the corneal facets, which are square in the crayfish, was determined

[2] $y = b\,x^k$. y = linear dimension of organ or cell; x = linear dimension of reference organ or the comparable dimension derived from the body weight $(W^{\frac{1}{3}})$; b = constant of proportionality; k = allometric coefficient, indicated by the slope when log y is plotted versus log x.

by tracing the outline of a suitable number (20–25) from the most distal part of the eye, again using camera lucida. The most distal facets are about three times as long on a side as those near the base of the eye. The average length on a side of the distal facets was recorded together with the data on eye diameter, abdomen length and breadth, and the body weight in grams.

Results

Respiratory rate

In Figure 1 is plotted resting metabolic rate versus body weight in grams, each scale being in logarithmic form. The straight line, drawn by eye, has a slope of

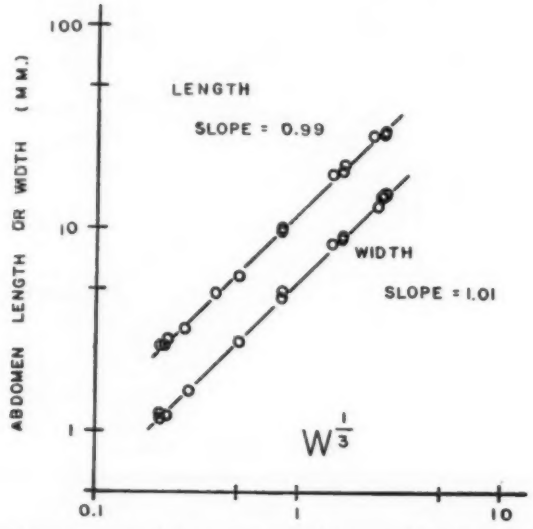

Figure 3. Abdomen length (upper plot) and abdomen width (lower plot) plotted against the cube root of the body weight in grams. Both coordinates are in log form. The equations of the lines are as follows: Abd. length (mm.) $= 12\ (W^{\frac{1}{3}})^{0.99}$. Abd. width (mm.) $= 5.5\ (W^{\frac{1}{3}})^{1.01}$.

-0.24, a value intermediate between 0 (metabolic rate independent of body weight) and -0.33 (metabolic rate following the surface law). Metabolic rate decreases from about 0.355 cc./hr./gm. at 10 mg. to 0.066 cc./hr./gm. at 10 gm., about a 5.4-fold decrease in rate, and considerably less than the 10-fold decrease expected on the basis of the surface law. Weymouth *et al.* (1944) reported a similar value for the exponent (-0.20) in a study of the metabolism of *Pugettia* and other marine crustacea. In *Procambarus*, the rate at 10 gm. (0.066 cc./hr./gm.) compares favorably with the respiratory rate reported for the European lake crayfish *Astacus leptodactylus* (0.07 cc./hr./gm. at 20 C.°) (Prosser *et al.*, 1950).

Muscle cell surface

Figure 2 represents the calculated surface concentrations obtained from average fiber diameters plotted against body weight in grams, again in double logarithmic form. The straight line, fitted by eye, indicates a slope of −0.22, indicating that growth of the muscle is accomplished by the enlargement of fibers, once formed, and the progressive addition of fibers throughout the weight range. By exploring the teased and stained preparations, it is possible to observe stages in fiber formation. Spindle-shaped myoblasts come to lie together in closely packed longitudinal groups, with subsequent loss of their cell boundaries, and a peripheral arrangement of the

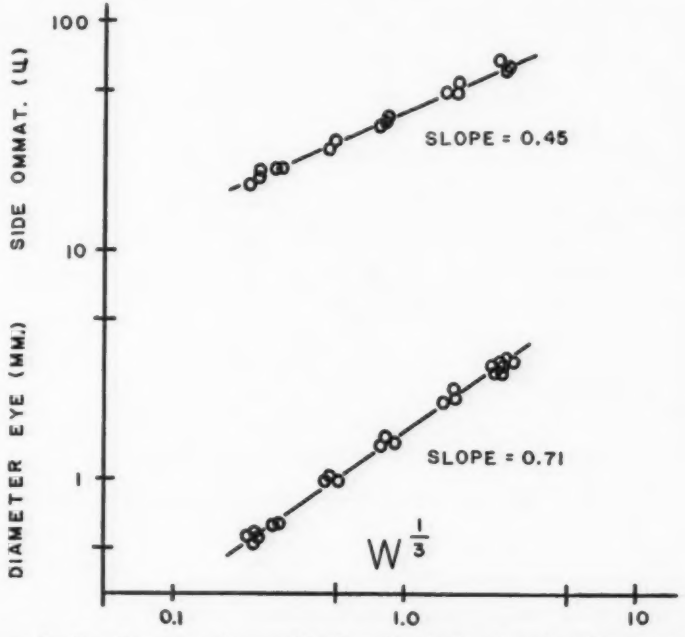

FIGURE 4. Eye diameter and ommatidia facet length plotted against the cube root of the body weight in grams. Both coordinates are in log form. The equations of the lines are as follows: side ommatidium (μ) = 39.8 $(W^{\frac{1}{3}})^{0.45}$, diameter eye (mm.) = 1.62 $(W^{\frac{1}{3}})^{0.71}$.

fiber nuclei. (For a discussion of the histogenesis of decapod skeletal muscle see Schneider, 1902.) It is not clear from the present study at exactly which stage in fiber growth the myoblasts stop contributing to the fiber. However, the spacing of the nuclei is much greater in large than in small fibers, indicating that the individual fiber receives all its nuclei while still relatively small.

Surface concentrations decrease from about 2240 cm.^{2}/cm.^{3} at 10 mg. to 500 cm.^{2}/cm.^{3} at 10 gm., corresponding to about a 4.5-fold increase in fiber diameters. The lower dotted line in Figure 2 indicates the 10-fold decrease which would occur if the fibers grew entirely by enlargement.

The observed 4.5-fold increase in fiber dimensions allows an indirect calculation of the increase in fiber numbers for a 1000-fold increase in weight. It may be assumed that fiber lengths are determined by the anatomical features of the origins and insertions, and accordingly undergo a 10-fold increase between 10 mg. and 10 gm. Since fiber diameters would also increase by a factor of 10 if their number remained constant, the relative increase in number of fibers is given by $(10/4.5)^2$ or 4.9. Thus, a weight increase of 1000-fold is accompanied by about a 5-fold increase in fiber number.

An alternative possibility to the addition of fibers during growth is that the abdomen might be markedly heterogonic with respect to the body as a whole. In

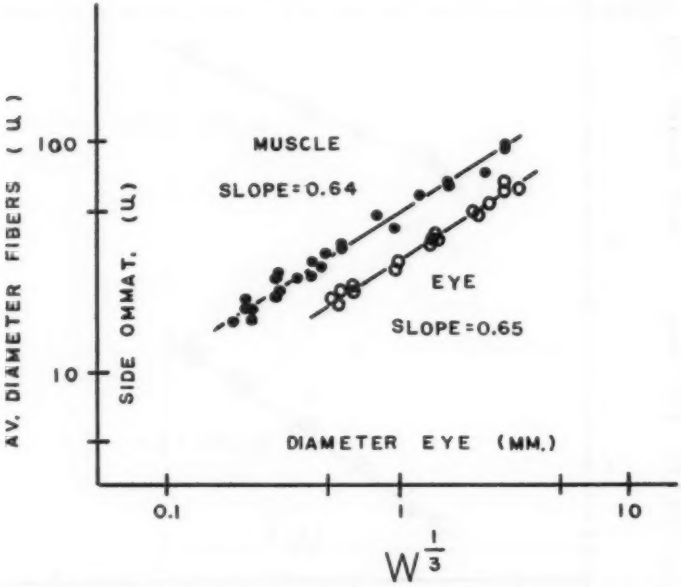

FIGURE 5. Average diameter muscle fibers plotted against the cube root of the body weight in grams (upper plot), and ommatidia facet length plotted against the diameter of the eye (lower plot). All coordinates are in log form. The equations of the lines are as follows: Av. fiber diam. (μ) = 49 $(W^{\frac{1}{3}})^{0.64}$; side ommatidium (μ) = 29.5 (diam. eye (mm.))$^{0.65}$.

order to test this possibility, abdomen length and breadth have been plotted against the dimensionally comparable quantity derived from the body weight (cube root of weight). The double log plot in Figure 3 indicates slopes for both length and breadth very close to unity, being 0.99 and 1.01, respectively. In other words, the relationship between fiber dimensions and body size is essentially identical to that between fiber dimensions and abdomen size. The abdomen and the whole body are isogonic.

Ommatidia and eye growth

The eye and its ommatidia are especially interesting in this connection, since each is markedly heterogonic with respect to body weight. Figure 4 illustrates

the allometric relationship between eye diameter (mm.) and ommatidia facet length (μ) when each is plotted against $W^{\frac{1}{3}}$. The slopes for each are less than unity, 0.71 for the eye, and 0.45 for facet length. The lower value for facet length indicates that growth of the eye is characterized by the progressive addition of receptor units. Knowing the allometric coefficient (slope) for each with respect to $W^{\frac{1}{3}}$, it is possible to calculate the allometric coefficient of ommatidia size with respect to eye size. This will be the ratio of the slopes or 0.45/0.71 which equals 0.635.[3]

Since average muscle fiber diameters increase systematically with body weight, it should also be possible to express this growth according to Huxley's allometric formulation. In Figure 5 are plotted both average fiber diameter (μ) against W (upper plot) and, on the basis of direct measurements, facet length (μ) against eye diameter (mm.). Fiber diameter indicates a slope of 0.64 and facet length a slope of 0.65, each value agreeing closely with the calculated slope (0.635) for facet length versus eye diameter obtained above.

Since the eye is a two-dimensional (surface) system of receptor units, the relationship between number of ommatidia and size of the eye will be essentially identical with that between number of fibers and size of the abdomen, in which the increase in number is also dependent on two-dimensional quantities (cross-sectional area of the abdomen and the fibers).

The close agreement of the allometric coefficients for the ommatidia-eye system and the fiber-body system indicates that the same basic mechanism of simultaneous addition and enlargement operates in each.

Discussion

It is clear, then, that a departure from the surface law of metabolism has been associated with a quantitatively similar departure in the manner of cell growth. Both metabolic rate and muscle surface concentration vary as the body weight raised to a power intermediate between -0.33 and 0. These observations are consistent with the view that the metabolic rate of the whole animal may be determined by the cell surface of the skeletal muscle. In addition, another organ system, the eye, indicates a manner of growth which is quantitatively comparable to that of the body as a whole.

Huxley (1932) has compiled a list of arthropod allometric coefficients drawn largely from Teissier's paper (Teissier, 1931). In the European walking stick (*Dixippus morosus*) values of the allometric coefficient are 0.48 and 0.37 for the eye and ommatidia diameters, respectively (measured against total length), giving a value of 0.77 (0.37/0.48) for ommatidia diameter versus eye diameter, and indicating a manner of growth comparable to that observed in the present study. Trager's analysis indicates that the tissues of the flesh fly larva (*Lucilia sericata*) increase in size solely as the result of cell enlargement (Trager, 1935). He also cites a number of earlier papers suggesting the growth of larval tissues by cell enlargement. Among these is a study by August Weismann (1864) who noted an

[3] $\log$ (facet length) $= 0.45 \log (W^{\frac{1}{3}}) + \log b$, and: $\log$ (eye diameter) $= 0.71 \log (W^{\frac{1}{3}}) + \log b'$. Solving each for $\log (W^{\frac{1}{3}})$: $\frac{\log \text{ (facet length)}}{0.45} - \log b = \log (W^{\frac{1}{3}}) = \frac{\log \text{ (eye diameter)}}{0.71} - \log b'$, and eliminating the common term: $\log$ (facet length) $= 0.45/0.71 \log$ (eye diameter) $+ \log b - \log b'$.

increase in size but not in number of muscle cell nuclei during the larval growth of muscid flies.

I would like to point out that the findings of the present study, as well as those cited by reference, are not in agreement wth Zeuthen's repeated statements to the effect that metazoan growth is characterized by an increase in number and not in size of cells (Zeuthen, 1953, 1955).

However, Zeuthen has made what may be a very pertinent observation when considered from the point of view developed here. Considering the animal kingdom as a whole, he points out that both in very small (less than a few mg.) and in very large (several kg.) animals, metabolic rate tends to change less markedly with body weight than in animals from the middle range of weight (Zeuthen, 1949). A possible explanation for this phenomenon may reside in a consideration of cellular structure. In very small animals a practical lower limit in cell dimensions may be reached so that smaller animals are composed of fewer rather than smaller cells. Similarly, there may also exist an upper limit on cell dimensions beyond which larger animals would be composed of more rather than proportionately larger cells.

Bertalanffy and co-workers (1951, 1953) prefer to attribute the phenomenon of size-dependent metabolism to "organismic factors." Since the phenomenon is expressed by the intact organism, there seems little doubt that organismic factors will play their part. However, cellular structure is not thereby precluded as a determining or, under standard conditions, a limiting factor in the expression of metabolism by the whole animal. The argument developed here points to a view of the organism as a highly integrated cell aggregate or collection of unit organisms (cells) whose growth, and hence structural, properties determine the performance of the animal proper. Virchow expressed a similar, if oversimplified, view of the organism in 1858 when he said, "Every animal appears as a sum of vital units, each of which bears in itself the complete characteristics of life." [4]

Summary

1. The resting metabolism of *Procambarus alleni* was determined over about a 4000-fold weight range. Metabolic rate was found to vary directly as the body weight raised to the − 0.24 power.

2. Abdominal muscle fiber diameters were determined over about a 4000-fold weight range. Calculated surface concentration was found to vary directly as the body weight raised to the − 0.22 power, providing evidence that the growth of the skeletal muscle is characterized by simultaneous addition and enlargement of fibers throughout the weight range.

3. Allometric analyses were made of fiber diameter, eye diameter, and ommatidia size with respect to the cube root of the body weight. The analyses indicated that the growth of the eye with respect to its constituent units is quantitatively similar to the growth of the abdomen with respect to its skeletal muscle cells.

4. The close similarity between the allometric coefficients of metabolic rate and surface concentration was taken as evidence that the metabolic rate of the whole animal may be dependent on the cell surface of the skeletal muscle.

[4] Quoted in introductory chapter to E. B. Wilson, The cell in development and heredity, 1925.

5. Evidence obtained from the literature was cited for other examples of the growth of arthropod tissues by cell enlargement.

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PHASE DIFFERENCE IN THE TIDAL RHYTHMS OF COLOR CHANGE OF TWO SPECIES OF FIDDLER CRAB [1]

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Persistent behavior patterns correlated with cosmic events have been reported for numerous animals. Brown, Fingerman, Sandeen and Webb (1953) recently reviewed the extensive literature dealing with persistent tidal rhythms in invertebrates. Bohn (1903) and Gamble and Keeble (1903, 1904) were the first investigators to report a rhythm with tidal frequency. A flatworm, *Convoluta roscoffensis,* was observed to emerge onto the surface of the sand at low tide and disappear into the sand as the tide rose. This rhythmic activity persisted in laboratory aquaria in the absence of contact with the tides.

Pigment in the melanophores of fiddler crabs, *Uca,* exhibits a daily rhythm of migration causing the animal to be dark by day and light by night. This daily rhythm persisted for at least several weeks in constant darkness in the laboratory without loss of synchrony with solar day-night. The frequency of this rhythm is independent of temperature between 6° and 26° C. (Brown and Webb, 1948).

Brown, Fingerman, Sandeen and Webb (1953) demonstrated a persistent tidal rhythm of migration of the pigment in the melanophores of *Uca pugnax.* The tidal rhythm of *Uca pugnax* expressed itself by a supplementary dispersion of the pigment in the melanophores one to three hours later than low tide. A low tide occurs twice every lunar day on the beaches near Woods Hole, Massachusetts, where the *Uca pugnax* were collected. The tidal rhythm is superimposed upon the daily rhythm and leads to a third type, a semilunar rhythm, because the daily and tidal rhythms are in the same phases with respect to one another only once every 14.8 days.

The phases of the tidal rhythm bore a definite relationship to the times of low tide in the native habitat of the *Uca pugnax.* Fiddler crabs collected from two diverse localities where the times of low tide differed by four hours showed in the laboratory persistent tidal rhythms in phase with the tidal changes of their respective original habitats. The tidal rhythms of the two groups of *Uca* were out of phase with each other by approximately four hours.

The frequency of the tidal rhythm of pigment migration in the melanophores of *Uca pugnax* is independent of temperature between 13° and 30° C. (Brown, Webb, Bennett and Sandeen, 1954). Hines (1954) demonstrated a tidal rhythm in the behavior of the melanophores in autotomized legs of *Uca pugnax.*

Fingerman (1955) found a tidal rhythm of migration of the pigment in the melanophores of the blue crab, *Callinectes sapidus.* The form of this tidal rhythm

[1] This investigation was supported by Grant No. B-838 from the National Institutes of Health.

was similar to the form of *Uca pugnax*. The *Callinectes* were collected in the vicinity of New Orleans, Louisiana, where the tides are chiefly diurnal. The tidal rhythm of *Callinectes* expressed itself through a supplementary dispersion of the melanin in the chromatophores about one hour after both the low and high tides, which occur 12.4 hours apart in a region with diurnal tides.

The current investigation was initiated in order to determine the character of the tidal rhythm of two species of *Uca* from a region of diurnal tides and to compare the rhythms of these *Uca* with the rhythm of the *Uca pugnax* which were collected in a semi-diurnal tidal habitat.

Materials and Methods

Adult male and female specimens of *Uca pugilator* and *Uca speciosa* were collected on the beach near the Gulf Coast Research Laboratory at Ocean Springs, Mississippi, and transported to an air-conditioned laboratory in New Orleans, Louisiana, for use in the observations to be described. From the evening of the day of collection until the end of the period of observation the crabs were maintained in darkness except for the few minutes required to make the observations. The temperature in the laboratory was 20° C. on the day the first *Uca* were collected. The temperature increased gradually throughout the spring and summer. On the day the last observations were performed, the temperature in the laboratory was 24° C. The *Uca* were kept in stainless steel aquaria containing sea water. The aquaria were inclined so that the bottom was not completely covered with sea water. The *Uca* were able to move into and out of the water. The sea water in the aquaria was changed every second day at 7:15 P.M.

The method of staging the chromatophores was the same as described by Brown, Fingerman, Sandeen and Webb (1953). The average chromatophore index of the pigment in the melanophores on the anterior aspect of the walking legs was determined with the aid of a stereoscopic dissecting microscope and microscope lamp hourly from 8 A.M. through 7 P.M. every second day. The chromatophore index of Hogben and Slome (1931) was employed to stage the melanophores. The most concentrated state of the pigment is described as stage 1, the most dispersed as stage 5, and the intermediate conditions as stages 2, 3, and 4.

Table I lists the times of high and low tide at Ocean Springs on the days observations were performed in the laboratory. Because of the astronomical conditions during this portion of the year the tides did not follow a consistent pattern and were not regularly spaced 12.4 hours apart. The times were obtained from the tide tables published by the Coast and Geodetic Survey, United States Department of Commerce.

Results

The tidal rhythm of Uca pugilator

On June 1, 1955, approximately 2000 *Uca pugilator* were collected. The crabs were divided into two groups and placed in darkness in the laboratory at 7 P.M. The animals from this collection will be referred to as the *Uca* of Series A. For the hourly determinations of the chromatophore index, 50 *Uca* were taken alternately from each lot. Series A was observed every second day from June 2 through

July 20, 1955. The hourly chromatophore indices obtained are presented in Table II.

The data obtained from the Series A *Uca* on June 12 through June 30 were used to prepare Figure 1. These data were selected because they illustrate the nature of the tidal rhythm. On June 12 (Fig. 1A) the peak of the curve was far to the right. Two days later on June 14 (Fig. 1B) the peak had shifted to the left. The peak then gradually shifted to the right again (Fig. 1C, D, E, F, and G). In Figure 1H (June 26) the peak was far to the right as in Figure 1A. As seen in Figure 1I (June 28) the peak had again shifted to the left just as in Figure 1B. The peak shifted day by day at the same rate as the tides. The shift of the peak from the

TABLE I

The times of low and high tide at Ocean Springs, Mississippi, on days observations were made

	High tide	Low tide
June 2	7:52 A.M.	7:11 P.M.
4	8:58	8:25
6	10:12	9:44
8	11:31	10:51
10	12:46	11:34
12	1:36 P.M.	11:27
14	6:46 A.M.	5:23
16	6:58	6:09
18	8:18	7:50
20	10:02	9:38
22	11:51	11:03
24	1:22 P.M.	11:20
26	6:14 A.M.	8:36
28	5:42	5:33
30	6:43	6:30
July 2	8:02	7:46
4	9:28	8:53
6	10:48	9:46
8	12:01	10:13
10	1:03 P.M.	9:46
12	4:59 A.M.	4:30
14	5:40	5:14
16	7:15	6:57
18	9:13	8:38
20	11:08	9:59

right to the left is due to the tidal influence making its appearance early in the morning. The tidal effect became progressively later in the evening until it had no ability to maintain a peak late in the day. The tidal rhythm was superimposed upon the daily dispersion of the pigment in the melanophores as has been described for *Callinectes sapidus* and *Uca pugnax*.

Figures 1H and 1I were obtained 14 days after Figures 1A and 1B, respectively. The 14 days required for the passage of the rhythm is the approximate duration of a semilunar rhythm.

Calculation of the exact relationship between the times of high and low tide and the phases of the tidal rhythm were impossible because of the peculiarity of the tides

at this time of the year. The general relationship between the time of the tide and the time of maximal tidal effect upon the melanophores previously observed with *Uca pugnax* and *Callinectes sapidus* holds in the results reported here. The maximal tidal effect occurred about two hours after the time of the tide.

To illustrate better the nature of this tidal rhythm Figure 2 and Table III have been prepared in the following manner from the data presented in Table II. The 12 periods of observation were divided into four periods of three hours each, 8–10 A.M., 11 A.M.–1 P.M., 2–4 P.M., and 5–7 P.M. The percentage of the total daily excursion that each three-hour period occupies was then calculated. The 12 hourly

TABLE II

The average melanophore index for each of the 12 daily observations of the Uca pugilator of Series A

	8 A.M.	9	10	11	12	1 P.M.	2	3	4	5	6	7
June 2	2.7	3.5	2.9	3.3	2.5	3.0	3.0	3.0	2.2	3.0	2.5	2.6
4	2.9	3.1	2.8	3.7	2.9	3.3	2.8	3.0	2.6	2.6	2.2	2.6
6	3.0	3.1	3.0	3.3	3.1	3.7	3.1	3.6	3.1	3.1	3.3	2.8
8	2.8	3.0	3.3	3.1	3.5	4.1	3.9	4.0	3.1	3.5	3.1	2.8
10	3.6	3.3	3.2	3.7	3.6	3.8	3.3	4.3	3.8	4.0	3.5	3.5
12	3.5	3.5	3.4	3.5	3.4	3.6	3.6	3.8	3.7	3.9	3.5	3.5
14	3.7	4.7	3.9	4.1	3.1	3.7	3.4	3.5	3.3	4.0	3.4	3.6
16	4.0	3.6	3.6	3.8	3.4	3.5	2.8	3.2	2.9	3.1	2.6	2.7
18	3.5	3.6	3.4	3.9	3.8	3.5	3.3	3.5	3.2	3.0	2.8	2.5
20	3.3	3.9	3.6	3.2	3.2	3.8	3.2	3.5	3.5	2.9	2.4	2.5
22	3.2	3.4	3.2	3.5	3.2	3.4	3.6	3.2	3.5	3.6	2.6	3.1
24	3.0	2.9	3.1	3.2	3.4	3.0	3.3	3.4	3.8	3.3	3.3	2.6
26	3.1	3.3	2.8	3.0	3.1	3.3	3.2	3.2	3.3	2.9	3.3	3.3
28	3.1	3.2	3.3	2.9	2.7	2.5	2.9	2.4	2.8	2.7	3.1	2.5
30	3.2	2.7	3.2	2.6	2.6	2.3	2.4	2.4	2.4	2.5	2.1	2.1
July 2	2.8	3.0	2.9	2.2	2.5	2.4	2.5	2.0	2.6	2.2	2.4	2.2
4	2.9	3.0	2.7	2.8	3.2	3.1	3.2	2.7	2.6	2.4	2.5	2.3
6	2.8	2.6	2.8	3.3	2.7	2.7	2.9	3.0	2.8	3.1	2.2	2.6
8	2.7	2.4	2.8	3.5	2.5	3.0	2.8	3.2	2.7	3.0	2.2	2.4
10	2.6	3.1	2.7	2.9	2.6	3.3	2.9	2.9	2.8	2.8	2.8	2.5
12	2.5	2.5	2.5	2.5	2.5	2.6	2.7	3.1	3.1	2.7	2.4	2.8
14	2.5	3.1	2.8	2.5	2.2	2.0	2.2	2.3	2.3	2.3	1.9	2.1
16	2.5	2.6	2.3	2.2	1.9	2.3	1.9	1.9	1.9	2.2	1.9	1.6
18	2.7	2.6	2.0	2.5	2.1	2.2	2.1	2.2	2.3	2.0	2.0	2.1
20	2.4	2.9	2.5	2.4	2.2	2.7	2.8	2.5	2.1	2.5	2.3	2.0

averages were summed and 12 subtracted from the total because if there had been no daily excursion of the pigment the sum would have been 12. Similarly, the average chromatophore indices for each three-hour period were summed and three subtracted from the total. The percentage of the total that each three-hour period occupied was then calculated. In Figure 2 the circles are the values calculated from the data of the *Uca pugilator* of Series A. The tidal maxima and minima progressed across the scale at a rate closely approximating the rate at which a tidal rhythm would be expected to progress. The parallel diagonal lines have been drawn through the tidal maxima at a distance of 14.8 days apart. Fourteen and eight-tenths days is the expected interval between days on which a low and a high

tide will occur at the same time of day at any given location where the tides are diurnal in nature.

Comparison of the tidal rhythms of Uca pugilator and Uca speciosa

On June 15, 1955, 1000 *Uca pugilator* (Series B) were collected. The *Uca* of Series B were handled and observed in the same manner as the *Uca* of Series A

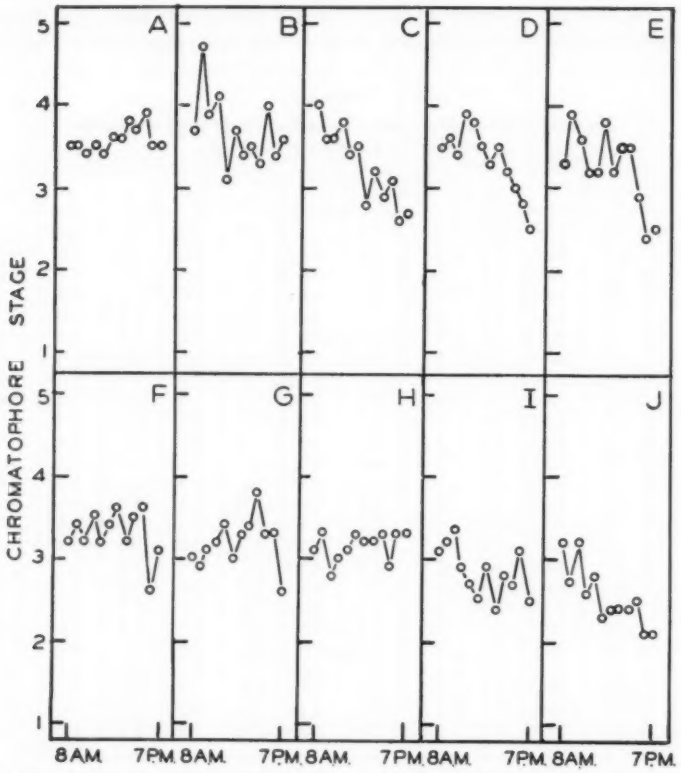

FIGURE 1. The daily pattern of the *Uca pugilator* of Series A observed every second day from June 12 (A) through June 30 (J).

with the one exception that all the *Uca* of Series B were maintained in one aquarium. Fifty *Uca* were observed at each reading. The data obtained from the observations of Series B were used in the preparation of Tables IV and V and Figure 2. The dots in Figure 2 are the data taken from Table V. As is evident from Figure 2, the *Uca pugilator* of both Series A and B were rhythmically similar although the *Uca* of Series B had been collected two weeks after the crabs of Series A. The tidal rhythm of the *Uca* in the laboratory during the first two weeks of June (Series A) must have progressed at the same rate as the rhythm of the *Uca pugilator* which

were on the beach at Ocean Springs 93 miles to the east during the same period of time (Series B).

Seventy-one specimens of *Uca speciosa* (Series C) were also collected at Ocean Springs on June 15. The *Uca* of Series A, B, and C were treated in an identical manner. Fifty of the *Uca speciosa* were observed at each reading. When the number of animals had decreased to 50, all of the remaining *Uca speciosa* were observed. The number of individuals in Series C gradually decreased until 42 remained on the last day of observation (July 14). The data obtained with Series C were used to prepare Tables VI and VII. The *Uca pugilator* (Series A and B)

TABLE III

The percentage of the daily melanin dispersion occurring at each of four periods of the day in Series A

	8–10 A.M.	11 A.M.–1 P.M.	2–4 P.M.	5–7 P.M.
June 2	27.5	26.1	23.4	23.0
4	25.8	30.7	24.0	19.6
6	23.3	27.1	26.0	23.7
8	21.6	27.3	28.4	22.7
10	22.5	25.6	26.6	25.4
12	24.0	24.3	26.2	25.6
14	28.7	24.4	22.2	24.7
16	30.1	28.3	21.7	19.9
18	26.8	29.3	25.0	18.9
20	28.9	26.7	26.7	17.8
22	24.7	25.8	26.5	23.0
24	22.8	25.1	28.5	23.6
26	24.0	24.8	26.0	25.2
28	29.9	23.1	23.1	24.0
30	32.6	25.2	22.5	19.8
July 2	32.2	23.2	23.2	21.5
4	26.2	28.5	25.7	19.6
6	24.2	26.5	26.5	22.8
8	23.1	28.3	26.9	21.7
10	24.7	26.5	25.6	23.3
12	22.6	23.1	29.7	24.6
14	33.9	22.8	23.5	20.4
16	33.3	25.8	20.5	20.5
18	29.0	25.7	24.3	21.0
20	27.7	24.9	25.4	21.9

were found to be six days out of phase with the *Uca speciosa* (Series C) as can be seen from Figure 3. In this figure sections A and B are the curves obtained from the *Uca pugilator* of Series A on June 26 and 28, respectively. Sections C and D represent the observations of the *Uca pugilator* of Series B on June 26 and 28. Sections E and F were taken from the observations of the *Uca speciosa* (Series C) for June 20 and 22, respectively. The curves for the *Uca pugilator* of both Series A and B were similar to one another as is evident from Figure 3. On June 26 the major peak of the curves from Series A and B was far to the right. On June 28 the major peak in Series A and B had shifted far to the left as the predominant tidal influence shifted from evening to early morning. A similar shift of peaks had oc-

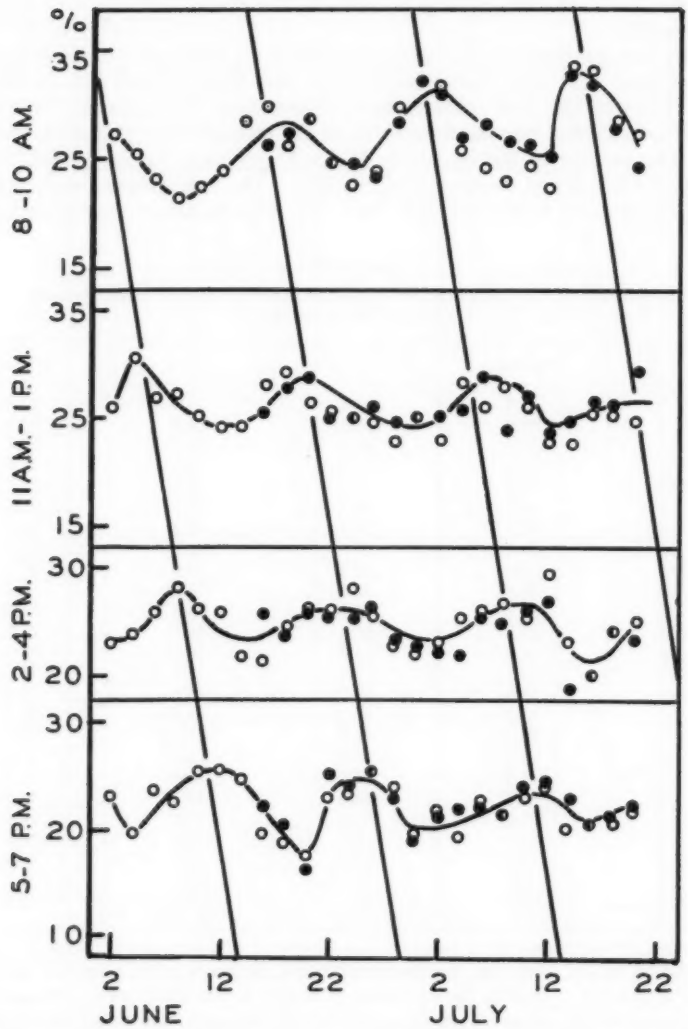

FIGURE 2. The relationship between the percentage of the daily melanin dispersion of *Uca pugilator* occurring at each of four periods during the day and the day of the month. Note the tidal maxima passing over the daily periods at the tidal rate of approximately 50 minutes per day. There is also a 14.8-day cycle, the interval between each of the parallel diagonal lines. The circles represent the *Uca pugilator* collected June 1 (Series A). The dots represent the *Uca pugilator* collected on June 15 (Series B).

TABLE IV

The average melanophore index for each of the 12 daily observations of Series B, Uca pugilator

	8 A.M.	9	10	11	12	1 P.M.	2	3	4	5	6	7
June 16	3.2	3.2	2.9	3.4	3.0	2.7	2.8	3.3*	3.1	2.7	3.0	2.6
18	3.6	3.7	3.3	3.6	3.7	3.4	2.9	3.4	3.3	3.1	2.8	2.7
20	3.8	3.9	3.8	3.8	3.7	4.0	3.4	4.3	3.0	2.9	2.5	2.4
22	3.2	3.5	3.6	3.6	3.3	3.5	3.6	3.5	3.6	3.8	3.4	3.2
24	3.2	3.7	3.6	3.6	3.6	3.8	3.4	3.5	3.9	3.8	3.3	3.2
26	3.3	3.4	3.6	3.5	3.6	3.5	3.8	3.6	3.8	3.7	3.7	3.4
28	3.8	4.0	3.7	3.6	3.5	3.4	3.2	3.3	3.5	3.4	3.2	3.2
30	3.7	4.0	3.6	3.6	3.0	2.9	2.9	3.0	2.9	2.6	2.8	2.5
July 2	4.0	3.7	3.7	3.4	3.4	3.2	3.2	3.0	2.9	3.1	2.7	2.9
4	3.5	3.6	3.5	3.8	3.9	3.6	3.3	3.0	3.0	3.2	2.9	3.1
6	3.9	3.3	3.6	3.5	3.4	2.6	3.1	3.5	3.4	3.2	3.0	2.7
8	3.5	3.9	3.5	3.9	3.6	3.5	3.6	3.4	3.4	3.1	3.3	2.9
10	3.8	3.5	3.4	3.2	3.3	3.3	3.8	3.6	3.2	3.4	3.5	3.0
12	3.5	3.2	3.1	3.2	3.0	3.1	2.9	3.6	3.8	3.5	3.0	2.9
14	3.5	3.5	3.4	3.1	2.8	2.6	2.3	2.8	2.2	2.9	2.7	2.6
16	3.6	3.2	3.1	3.0	2.9	2.9	2.6	2.6	2.4	2.3	2.5	2.6
18	3.4	3.4	3.0	3.2	3.0	3.1	3.0	3.7	3.1	2.8	2.5	2.8
20	3.1	3.0	3.3	3.6	3.2	3.9	3.3	3.0	2.9	3.1	2.8	3.0

curred in the *Uca speciosa* of Series C six days prior to the shift in the animals of Series A and B.

Figure 4 was prepared in the same manner as Figure 2 to illustrate better the tidal phase difference between the two species. In Figure 4 the circles are the

TABLE V

The percentage of the daily melanin dispersion occurring at each of four periods of the day in Series B

	8–10 A.M.	11 A.M.–1 P.M.	2–4 P.M.	5–7 P.M.
June 16	26.4	25.5	25.9	22.2
18	27.6	28.0	24.0	20.4
20	28.8	28.8	26.1	16.3
22	24.5	24.8	25.8	24.8
24	24.5	26.1	25.5	23.9
26	23.6	24.6	26.5	25.2
28	28.5	25.2	23.5	22.8
30	32.6	25.5	22.7	19.2
July 2	30.9	25.7	22.4	21.0
4	26.8	29.2	22.2	21.8
6	28.7	23.9	25.7	21.7
8	26.7	27.0	25.0	21.3
10	26.6	23.4	26.2	23.8
12	25.4	23.5	27.2	23.9
14	33.0	24.6	19.2	23.2
16	31.8	26.7	21.2	20.3
18	28.3	26.3	24.2	21.3
20	24.4	29.3	23.7	22.5

data obtained from the crabs of Series A and the dots are the data from the crabs of Series C. The tidal maxima of Series C occurred six days prior to the tidal maxima of Series A at all times of day. The curves of Series C had no predominant peak after the crabs had spent one week in darkness because the melanin in the chromatophores of the *Uca speciosa* stayed nearly maximally dispersed throughout the day, rendering difficult an exact determination of the date on which the tidal maxima and minima of each portion of the day occurred. The identical situation was observed with *Uca pugnax* (Brown, Fingerman, Sandeen and Webb, 1953).

The tidal difference of six days in Figure 4 has been expressed as 4.9 hours. The latter value was determined from calculations based on the fact that the tides advance 48.8 minutes per day.

Inspection of the beach at Ocean Springs showed that the burrows of the *Uca pugilator* were found only in the sand of the open beach, whereas the burrows of

TABLE VI

The average melanophore index for each of the 12 daily measurements of Series C, Uca speciosa

	8 A.M.	9	10	11	12	1 P.M.	2	3	4	5	6	7
June 16	2.8	3.7	3.7	4.0	4.1	3.7	3.6	3.8	3.6	3.4	3.4	3.0
18	3.7	3.7	3.8	3.6	4.1	4.4	4.4	4.6	4.4	4.3	4.1	3.3
20	3.5	4.3	4.3	4.5	4.5	4.5	4.7	4.7	4.7	4.6	4.6	3.9
22	4.4	4.8	4.8	4.8	4.6	4.6	4.5	4.4	4.5	4.6	4.5	4.1
24	4.3	4.7	4.7	4.6	4.8	4.7	4.6	4.6	4.6	4.5	4.2	4.2
26	4.4	4.6	4.6	4.6	4.4	4.5	4.4	4.5	4.4	4.5	4.5	4.3
28	4.4	4.7	4.8	4.8	4.8	4.8	4.8	4.6	4.8	4.6	4.6	4.4
30	4.6	4.7	4.7	4.6	4.6	4.7	4.6	4.6	4.5	4.6	4.4	4.5
July 2	4.0	4.3	4.7	4.7	4.7	4.8	4.7	4.8	4.8	4.7	4.7	4.6
4	4.6	4.8	4.6	4.7	4.7	4.7	4.6	4.7	4.7	4.7	4.4	4.4
6	4.8	4.7	4.8	4.8	4.7	4.8	4.7	4.8	4.7	4.7	4.7	4.6
8	4.7	4.7	4.9	4.8	4.8	4.7	4.8	4.6	4.6	4.7	4.5	4.4
10	4.5	4.6	4.7	4.7	4.7	4.6	4.7	4.5	4.6	4.5	4.4	4.3
12	4.7	4.8	4.8	4.8	4.7	4.6	4.6	4.7	4.7	4.5	4.7	4.3
14	4.7	4.9	4.8	4.9	4.8	4.7	4.6	4.5	4.6	4.5	4.5	4.3

the *Uca speciosa* were found only in the marsh grass from the high tide mark to the open sand. Figure 5 is a diagrammatic representation of the section of the beach at Ocean Springs where both species were collected.

Rarely did a *Uca speciosa* venture out of the marsh grass onto the open sand at low tide. On the other hand, at low tide the *Uca pugilator* left their burrows and moved to the water's edge to feed. The adaptive advantage of the tidal rhythm is thought to lie in the association between the time of low tide and the time at which the *Uca* leave their burrows to feed. The *Uca* at Ocean Springs are reported to leave their burrows to feed at low tide both day and night. Fiddler crabs living in a region of semi-diurnal tides leave their burrows to feed only when low tide occurs during daylight. If the fiddler crabs at Ocean Springs did not feed during a low tide at night, they would have to go without food for intervals of approximately 10 days when low tides occurred at night.

To explain the tidal difference between the two species of *Uca* the following

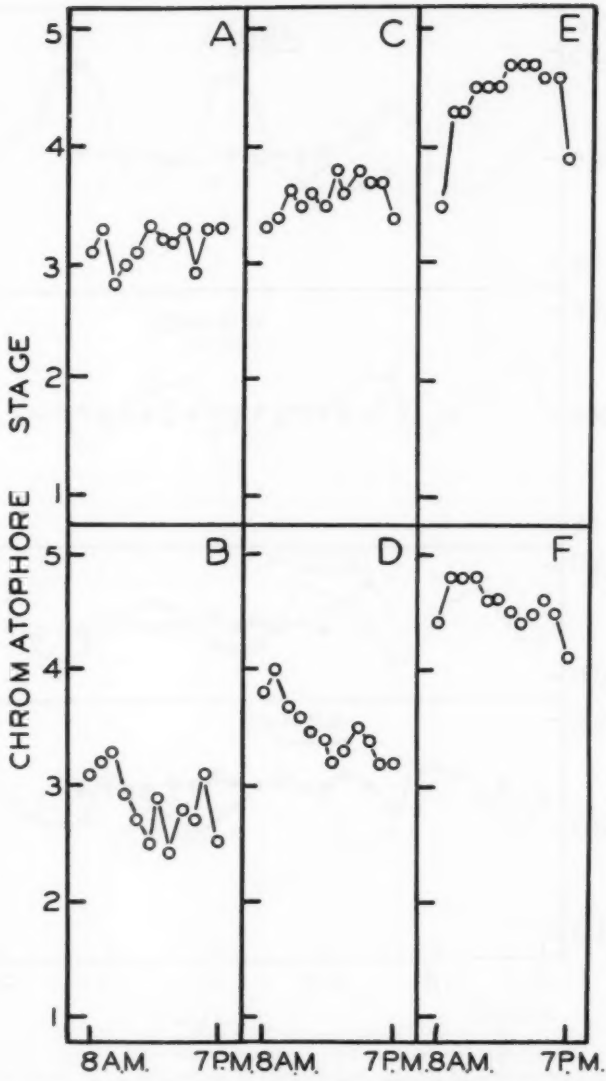

FIGURE 3. The daily patterns obtained on June 26 and 28 for Series A and B (*Uca pugilator*) and on June 20 and 22 for Series C (*Uca speciosa*). A and B in the figure are from Series A; C and D from Series B; E and F from Series C.

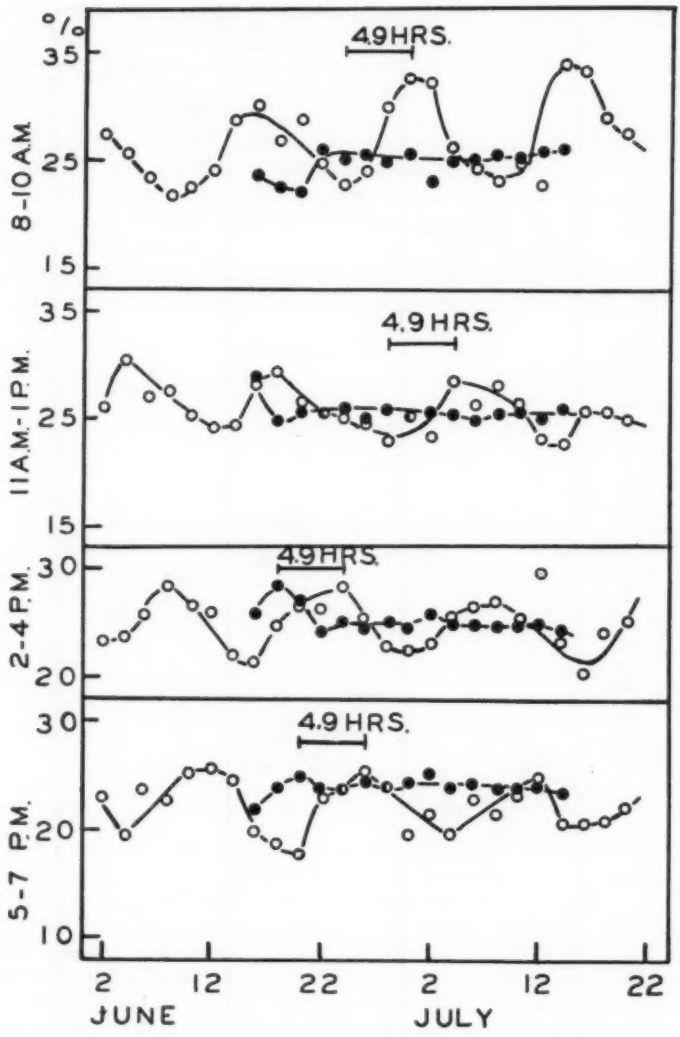

FIGURE 4. The relationship between the percentage of the total melanin dispersion occurring at each of four periods of the day and the day of the month. The circles represent the *Uca pugilator* collected on June 1 (Series A). The dots represent the *Uca speciosa* collected June 15 (Series C). The tidal curves of Series C are displaced six days to the left of the curves of Series A. In a tidal cycle, a displacement of six days is equivalent to 4.9 hours.

TABLE VII

The percentage of the daily melanin dispersion occurring at each of four periods of the day in Series C

	8–10 A.M.	11 A.M.–1 P.M.	2–4 P.M.	5–7 P.M.
June 16	23.4	28.6	26.0	22.1
18	22.5	25.0	28.6	24.0
20	22.3	25.7	27.2	24.8
22	25.8	25.8	24.4	23.9
24	25.2	26.1	25.4	23.3
26	25.4	25.2	24.7	24.7
28	24.7	25.9	25.4	24.0
30	25.5	25.3	24.8	24.4
July 2	23.0	25.7	26.0	25.3
4	25.2	25.5	25.2	24.1
6	25.2	25.2	25.0	24.6
8	25.6	25.6	24.9	24.0
10	25.2	25.7	25.2	23.8
12	25.7	25.3	25.1	24.0
14	26.0	26.0	24.4	23.5

hypothesis was developed. As the water begins to recede following a high tide, in effect a local low tide occurs for the *Uca speciosa* in their burrows among the marsh grass higher on the beach. This local low tide would occur earlier higher on the beach than would the low tide for the *Uca pugilator* in their burrows in the sand. The species living among the grass would, therefore, be free to feed earlier than the

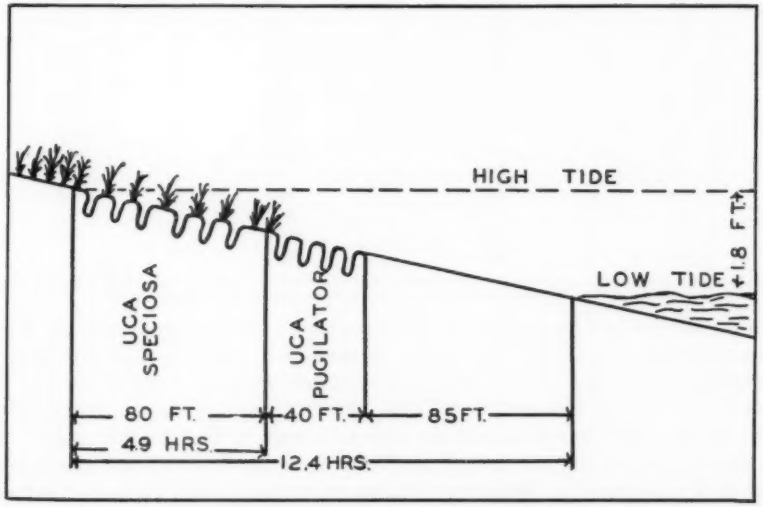

FIGURE 5. A diagrammatic representation of the section of the beach at Ocean Springs, Mississippi, where both species of *Uca* were collected.

species living in the sand. The earlier occurrence of a low tide in the marsh grass might, therefore, account for the *Uca speciosa* having a tidal rhythm 7.5 hours in advance of the tidal rhythm of the *Uca pugilator* whose burrows are in the sand.

To test this hypothesis measurements of the portion of the beach where both species were collected were made at low tide. To avoid bias, the measurements were made by an impartial local resident who was present when the collections were made on June 15. This resident was given a sketch of the beach. On this diagram were indicated the regions of the beach to be measured. He did not know how the data he obtained were to be treated nor did he know a 4.9 hour tidal difference be-

TABLE VIII

The amplitude of the daily rhythm of animals maintained in darkness. The amplitude is expressed as the total of the average stages for the 12 daily measurements of the melanophores

	Series A	Series B	Series C
June 2	34.2		
4	34.5		
6	38.2		
8	40.2		
10	43.6		
12	42.9		
14	44.4		
16	39.2	35.9	42.8
18	40.0	39.5	48.4
20	39.0	41.5	52.8
22	39.5	41.8	54.6
24	38.3	42.6	54.5
26	37.8	42.9	53.7
28	34.1	41.8	56.1
30	37.0	37.5	55.1
July 2	29.7	39.2	55.5
4	33.4	40.4	55.6
6	33.5	39.2	56.8
8	33.2	41.6	56.2
10	33.9	41.0	54.8
12	31.9	38.8	55.9
14	28.2	34.4	55.8
16	25.2	33.7	
18	26.8	36.0	
20	29.3	38.2	

tween the species had been found in the laboratory. The measurements have been incorporated into Figure 5. The width of the beach from the high tide mark to the low tide mark is 205 feet. The distance from the high tide mark to the first *Uca pugilator* burrows in the sand is 80 feet. Twelve and four-tenths hours are required for the water to move from the high tide mark to the low tide mark. The time required for the water to recede the 80 feet from the first *Uca speciosa* burrows to the first *Uca pugilator* burrows was then calculated. This value is 4.9 hours, close to the result observed in the laboratory for the tidal difference in hours between the two species.

A semilunar rhythm of amplitude of the daily pigment dispersion

The sums of the twelve daily average chromatophore indices for Series A, B, and C have been compiled in Table VIII and depicted in Figure 6. The amplitudes for the three series increased from the outset of the observations. The amplitude of the daily pigmentary rhythm of the *Uca pugilator* of both Series A and B

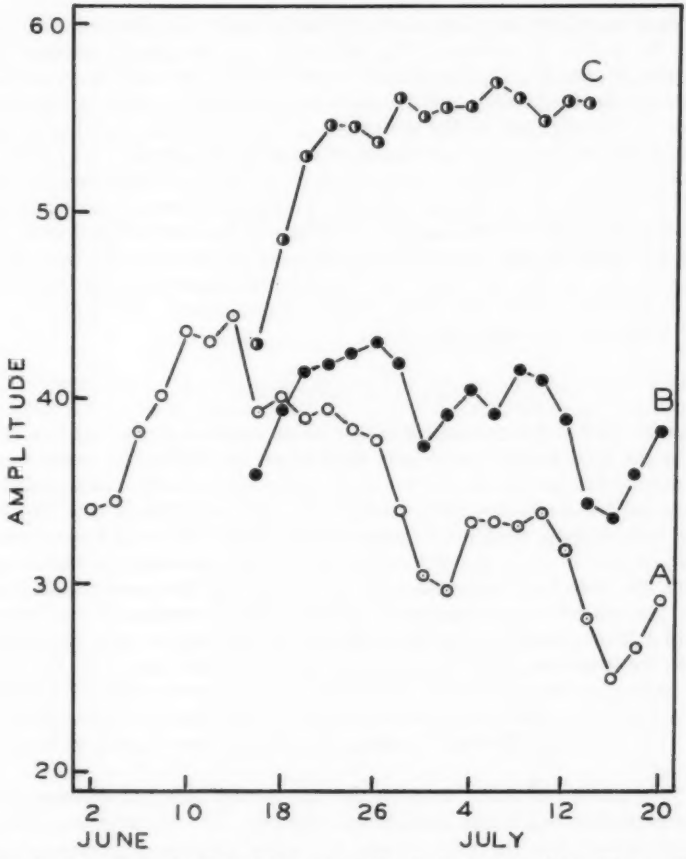

FIGURE 6. The amplitude of the daily patterns for Series A, B, and C. The amplitude has been expressed as the sum of the 12 average chromatophore stages determined every second day for each series. Series A and B are *Uca pugilator*. Series C is *Uca speciosa*.

then decreased noticeably. Increases and decreases followed one another. Closer inspection showed this increase and decrease of amplitude was semilunar in nature. The minima occurred 15 days apart which is the approximate frequency of a semilunar rhythm. Furthermore, these minima were observed on the same days that

the tidal maxima occurred in the 8–10 A.M. portion of the day (Figs. 2, 4, and 6). Closer inspection of the data from Series C also disclosed a semilunar rhythm of amplitude which was not as pronounced as the rhythm of Series A and B because of the tendency of the melanin in the chromatophores of the *Uca speciosa* to remain nearly maximally dispersed after a week in darkness. The minima of Series C occurred 5.5 days ahead of the minima of Series A and B, again demonstrating the phase difference between the species.

The days on which the minima of amplitude occurred coincided with the days on which the 8–10 A.M. portion of the daily curve was maximal (compare Figures 2 and 4 with Figure 6). To produce a maximum at 8–10 A.M. the P.M. tide must progress late into the evening and therefore has no ability to cause a supplementary dispersion of the pigment in the afternoon. The A.M. tide, 12.4 hours after the P.M. tide, is still too early to have an effect upon the amplitude. The daily rhythmicity alone keeps the pigment semi-dispersed in the late afternoon on the days with a late evening tide. As the A.M. tide progresses through the early morning hours toward the center of the day the amplitude of the entire daily curve is raised by the tidal influence and the 8–10 A.M. portion of the curve is maximal. The amplitude begins to decrease again as the tide progresses into the late afternoon and early evening. Then the daily rhythm alone causes the dispersion of the melanin during the morning hours.

General Discussion

The tidal rhythm determined for both *Uca pugilator* and *Uca speciosa* is similar in form to the tidal rhythm previously determined for *Callinectes sapidus* and *Uca pugnax* despite the fact the specimens of the latter species were taken from a region exhibiting only semi-diurnal tides. Originally this endogenous tidal rhythm was probably induced by a rhythmic environmental factor with a 12.4 hour frequency. This tidal rhythm has now become such a deep-rooted phenomenon that it persisted even after the crabs had been placed in an environment that was constant with respect to the rhythmic environmental factors. The necessity of the two events spaced 12.4 hours apart having been similar to one another was apparently secondary to the frequency which became impressed upon the crabs. If the frequency were of secondary importance to the nature of the events, then *Uca* living in a habitat with diurnal tides would have exhibited a rhythm with a frequency which is twice that observed; the tidal maxima would have been found to be 29.5 days apart.

The tidal phase difference between the two species living at different levels on the same section of beach was entirely unexpected. The occurrence of this difference demonstrates that the tidal rhythm has more adaptive significance than was suspected previously. The phases of the tidal rhythm are apparently set by the tides within an extremely limited strip of beach. According to the authorities at the Gulf Coast Research Laboratory only within the past year have the *Uca* invaded the portion of the beach where they were collected. The phases of their tidal rhythms were possibly reset in accordance with the influence of the local tides near their burrows within the past year.

The morphological difference in the melanophore pigment pattern of the two

species may be a partial explanation for the semilunar rhythm of daily amplitude being more apparent in *Uca pugilator* than in *Uca speciosa*. When the pigment in the melanophores of *Uca pugilator* was completely dispersed (stage 5) the melanophores were still discrete entities. Light portions of the exoskeleton could be seen between the melanophores. However, when stage 5 was attained by the melanophores of *Uca speciosa* the melanophores lost their individuality and the entire leg was homogeneously black. How this difference in morphology is translated into the difference in behavior is not readily apparent.

The chromatophore behavior of *Uca speciosa* is similar to the pigmentary behavior of *Uca pugnax*. The melanophores of both species darkened the entire leg when the pigment was completely dispersed. The chromatophores of both species also tended to remain completely dispersed throughout the day after a similar length of time in darkness.

The tidal rhythm of the *Uca pugilator* of Series A was determined in the laboratory over a period of 48 days which is the longest interval any group of *Uca* has ever been observed. Throughout this period there was no indication that the frequency of the tidal rhythm was changing.

Summary and Conclusions

1. The pigment in the melanophores of the fiddler crabs, *Uca pugilator* and *Uca speciosa*, showed a persistent tidal rhythm with a frequency of 12.4 hours.

2. The nature of the tidal rhythm of both species was similar to the tidal rhythms previously reported for the fiddler crab, *Uca pugnax*, and the blue crab, *Callinectes sapidus*.

3. No detectable difference in the character of the pigment migration was apparent at times of low and high tide which occur 12.4 hours apart where these two species of *Uca* were collected.

4. The tidal rhythm of *Uca speciosa* was six days behind the tidal rhythm of *Uca pugilator*. This difference is equivalent to 4.9 hours in a tidal cycle.

5. An hypothesis is presented to explain this tidal difference. The difference in the habitat of the two species of *Uca* on the same beach, the width of the beach, and the time required for the water to recede from the burrows of the *Uca speciosa* to the burrows of the *Uca pugilator*, which live at a lower level of the beach, are the factors which have been considered in formulating the hypothesis.

6. A semilunar rhythm of the amplitude of the daily dispersion of the pigment in the melanophores of both species was found.

7. The possible adaptive significance of the phase difference of the tidal rhythms of the two species is discussed.

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THE LOCI OF CONTACT CHEMORECEPTORS INVOLVED IN FEEDING REACTIONS IN CERTAIN LEPIDOPTERA [1]

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In our review (1949) of literature on the loci of contact chemoreceptors of insects we listed 10 species of butterflies which had been shown experimentally to have contact chemoreceptors on the tarsi. It was assumed that these insects also had receptors on the mouth-parts or in the mouth, but this had not been shown experimentally. Up to that time, four species of moths had been tested for possible possession of tarsal chemoreceptors and found not to possess them. At that time, we tested three species of butterflies, two of which had been shown previously to have tarsal chemoreceptors, located these more exactly and found that the terminal part of the proboscis in each is sensitive to chemical stimulation by solutions. The antennae and palpi were found not to be loci of contact chemoreceptors sensitive to sucrose solutions. The contact chemoreceptors on the tarsi and proboscis are involved in feeding reactions, and these should probably be so designated to differentiate them from chemoreceptors possibly involved in mating (Roth and Willis, 1952) or oviposition (Dethier, 1947).

As an extension of the earlier work, we have located the trophic contact chemoreceptors of four species of butterflies, only one of which has been tested previously, and two species of moths, neither of which has been tested previously.

Materials and Methods

The following species of butterflies were studied. The scientific names are those given by Klots (1951).

Family: Satyridae
Cercyonis pegala—The Wood Nymph or Grayling
subspp. *alope* and *nephele*
20 individuals, captured in the field
Lethe eurydice—The Eyed Brown
3 individuals, captured in the field
Family: Nymphalidae
Speyeria cybele—The Great Spangled Fritillary
2 individuals, captured in the field

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Limenitis arthemis—The White Admiral
2 individuals, captured in the field

The following species of moths were tested. The scientific names are those given by Borror and Delong (1954).

Family: Amatidae (Syntomidae, Ctenuchidae, Euchromiidae)
Ctenucha virginica
22 individuals, captured in the field
Scepsis fulvicollis—The Yellow-collared Scape Moth
15 individuals, captured in the field

In the first and last two species, these were approximately half males and half females. *Cercyonis pegala* was found by Anderson (1932) to have tarsal chemoreceptors; the others have not previously been tested. Reporting results obtained with the small number of individuals of the last three butterflies in the list is justified only because the responses were so clear-cut and so similar to those of the other related species. The moths are day-flying and feed on nectar, and thus differ in habits from the usual moths. They have well developed proboscides.

The butterflies were immobilized for testing as described by Minnich (1921, 1922a, 1922b) by clamping the wings together with spring clothes-pins. The moths were mounted by fixing the dorsum of the prothorax and the wings to wax blocks on the ends of glass rods, as described by Frings (1947). Thus fixed, the animals could easily be handled and observed.

The contact chemoreceptors were located as described previously (Frings and Frings, 1949). Series of tests of variable duration and number were made each day on the fixed animals. Before each series the insects were given all the water they would drink. These butterflies and moths proved to be avid drinkers, and often kept the proboscis extended for more than 10 minutes. A 1 *M* sucrose solution in water was used for most of the tests, and contact of this with the receptors elicited uncoiling of the proboscis and feeding if allowed. The proboscis response could thus be used as the index of reception. Temperatures were uncontrolled and varied from 18°–25° C.

For general location of the receptors, the part to be tested was either immersed in the sucrose solution in a small dish or the solution was brought to the part on small artists' brushes. Each test with the solution was preceded by one or more control tests with water. If at any time the insects responded to water, they were allowed to drink until sated, were thereupon put aside for a time, and were retested later.

For more specific location of the receptors, two methods were used. Local stimulation of suspected parts with droplets of sucrose solution on finely drawn, glass microneedles was used with uninjured insects. These tests were also preceded by control tests with water. Operations on the animals for removal of suspected or known loci and later testing were also performed, with paired controls similarly treated except for the removal of parts. Generally, the insects were unanaesthetized for these manipulations. The tests were carried out under a binocular dissecting microscope at up to 30 × magnification.

For microscopic examination of possible end-organs, structures were removed to 70% ethyl alcohol, transferred to 95% alcohol and mounted on slides in Diaphane.

Results

In these butterflies, the rudimentary fore-legs characteristic of the Nymphaloids, the palpi, and the antennae seem to lack receptors for sucrose. The antennae are quite sensitive to water vapor if the animals are thirsty. Receptors are present on the ventral sides only of the tarsi of the mesothoracic and metathoracic legs and on the distal portion of the proboscis, extending about ¼ of the length of the proboscis from the tip. With *C. pegala* and *L. eurydice,* but not with the other two species, local stimulation techniques showed that the three distal segments of the mesothoracic and metathoracic tarsi have receptors and the basal segment does not. The second segment of the mesothoracic tarsus in *C. pegala* definitely has the receptors along its entire length, while that of *L. eurydice* has them only at the distal end. Tests on the second segment of the metathoracic tarsus of *C. pegala* gave inconclusive results, while tests on this segment of *L. eurydice* gave definitely negative results. A total of 51 paired tests (H_2O vs. sucrose solution) were made in 10 days.

Ctenucha virginica proved to be an easy animal to study. A total of about 250 paired tests were made in 10 days. All the experimental methods were used. The palpi lack contact chemoreceptors mediating proboscis extension and the antennae seemed to lack them in the early tests. All the tarsi possess the organs. On the prothoracic and mesothoracic tarsi they are on the ventral sides of all five of the tarsal segments, but not on the distal parts of the tibiae. On the metathoracic tarsi, the receptors are on the ventral sides of the three terminal segments, probably on the second segment, and probably not on the basal segment and the tibia. The distal ¼ to ⅕, only, of the proboscis proved to be sensitive when tested with glass microneedles bearing 1 *M* sucrose solution after control tests with water. When the tarsi or the tip of the proboscis was touched with a 1 N NaCl solution, the animal drew the proboscis into a tighter coil. If the animal was feeding on sucrose solution, touching the tip of the proboscis with NaCl solution caused immediate recoiling of the proboscis. Thus these receptors allow differentiation between acceptable and unacceptable solutions.

Scepsis fulvicollis also proved to be easy to test, and all the methods for locating the receptors were used. A total of about 400 pairs of tests were made in 16 days. Two additional methods were also used with these. First, the insects were tested with saturated sucrose solution as well as 1 *M* solution. Second, after tests on the legs had been made, all the legs were removed and the antennae and palpi tested. In all the previous tests, the antennae and palpi were tested with the animals intact or with only the prothoracic or prothoracic and mesothoracic legs removed.

The palpi seem not to bear receptors mediating the proboscis response. The ventral surfaces of the three pairs of tarsi bear end-organs. On the fore-leg, the receptors are on all five segments of the tarsus, but not on the tibia. On the mid-leg, the distal four segments of the tarsus definitely bear receptors. When tested with 1 *M* sucrose solution, the basal segment seemed not to bear the receptors, but when tested with saturated sucrose solution definite positive responses were obtained. The tibia seemed not to have receptors. The hind-leg showed a similar situation. When tested with 1 *M* sucrose solution, only the three distal segments of the tarsus seemed to have receptors, but when tested with saturated sucrose solution all five of the segments proved to bear them. The tibia again seemed not to have these receptors. The distal ⅕ to ⅙ of the proboscis also proved sensitive to

sucrose solutions; uncoiling was elicited on contact with a needle moistened with the sucrose solutions. The other parts of the proboscis could be touched with sucrose solutions without eliciting uncoiling. NaCl solution, as in *Ctenucha*, elicited a rejection reaction.

The situation with respect to the antennae in *Scepsis* was interesting. As long as the animals had tarsi, the antennae seemed to lack the receptors. Only occasionally would the insect uncoil the proboscis when sugar-water touched the antennae, and usually the insect would then take water and afterward be negative to sugar-water. If only the hind tarsi or even one hind tarsus alone were present, the reaction to antennal contact indicated lack of receptors. When all the legs were removed, however, the antennae seemed receptive, and the differential response to

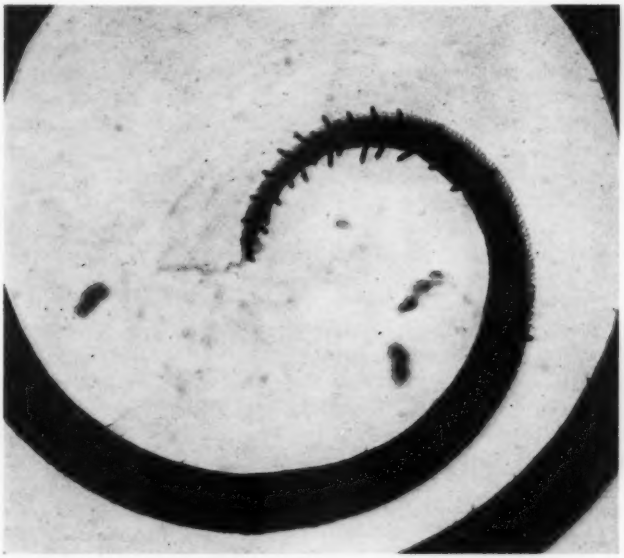

FIGURE 1. Terminal portion of proboscis of *Ctenucha virginica* (×70).

sucrose solution became almost perfect. By the time that this discovery was made, the individuals of the other species had died, and it was impossible to test them similarly.

The previous experiments were made during the summer of 1955. In January, 1956, adults of *Ctenucha virginica* were obtained from caterpillars reared from eggs laid during the past summer. Six of the adults (3 ♂♂ and 3 ♀♀) were tested and found to have antennal contact chemoreceptors. When either or both of the first two pairs of legs were present, no consistent reactions were obtained on stimulation of the antennae with sucrose solutions. When the first two pairs of legs were removed, leaving only the metathoracic legs, stimulation of the antennae with sucrose solutions consistently evoked the feeding response. Smyth (personal

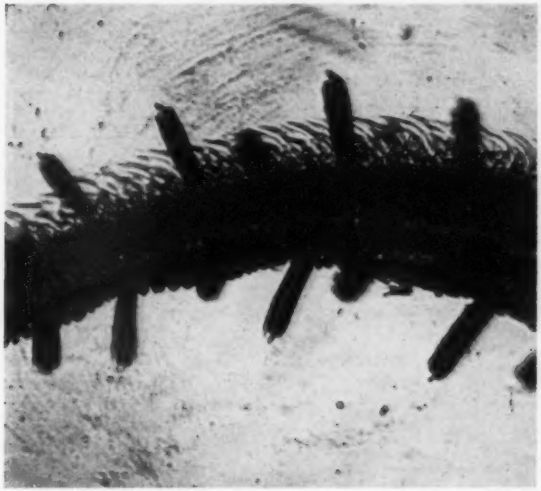

FIGURE 2. Section of terminal portion of proboscis of *Ctenucha virginica* (× 350) to show probable chemoreceptive sensilla. The convex side has a rather dense layer of non-sensory epicuticular "hairs."

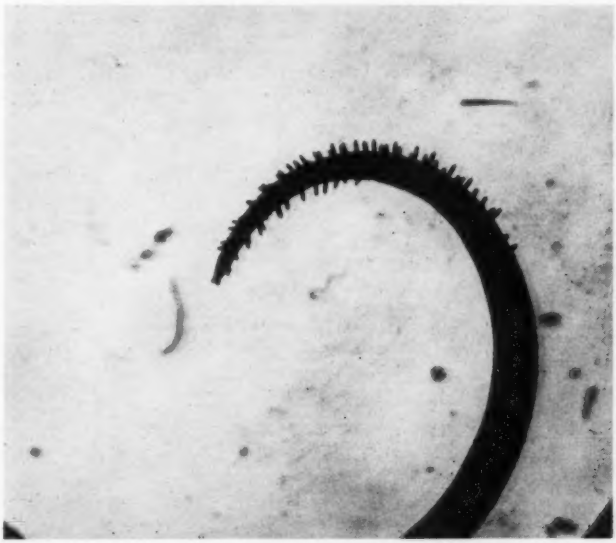

FIGURE 3. Terminal portion of proboscis of *Scepsis fulvicollis* (× 70).

communication) has recently recorded discharges from receptors on the antenna of *Scepsis* when they were stimulated with sucrose solutions, thus confirming electrophysiologically the results of the behavioral experiments.

Morphological studies on the tarsi of these Lepidoptera have not enabled us to select any particular sensilla as the end-organs. There are present, as in *Danaus plexippus* (Frings and Frings, 1949), short, thin-walled trichoid sensilla on the ventral surfaces of the tarsi among other stouter hairs and spines. These could be the receptors, but there is no proof of this.

On the proboscis of the moths studied, at the terminal portion found by experiments to be sensitive, there are many large sensilla (Figs. 1–4) that are almost certainly the contact chemoreceptors. These have a peculiar structure, appearing something like candles. There is a shaft, which is polygonal in cross-section, 6–7-

FIGURE 4. Section of terminal portion of proboscis of *Scepsis fulvicollis* (× 350) to show probable chemoreceptive sensilla. The epicuticular "hairs" on the convex surface are shorter than in *Ctenucha*.

sided in *Ctenucha* and 5–7-sided in *Scepsis*, with the angles seemingly thickened. This is capped with a thin-walled nipple-shaped structure, around the base of which the thickened corners of the shaft extend as points. In *Ctenucha* these range in size from 45–50 μ long and 12 μ thick at the proximal end of the group to 35–40 μ long and 12 μ thick near the tip, most being 40–45 μ long and 12 μ thick. At the tip there is a small group of shorter organs 20–25 μ long and 12 μ thick. In *Scepsis* the organs are somewhat smaller: 40–45 μ long and 9 μ thick at the proximal end decreasing regularly in size toward the tip to 25–30 μ long and 7 μ thick, most being 30–40 μ long and 7 μ thick. The organs in the group at the tip measure 15–18 μ long and 7 μ thick. The exact designation that one should give to these sensilla is not clear. Are they modified trichoids or basiconics? Since no other obvious sensilla appear at this region of the proboscis and these correspond in distribution

with the experimentally determined sensitive region, they are probably the end-organs.

Among the butterflies, we have examined only the proboscis of *C. pegala*. The distal ⅕ of it bears similar organs, 30–35 μ long and 7 μ thick, varying little in size throughout. These correspond in position also with the experimentally determined contact chemoreceptors and are thus probably the end-organs.

Discussion

The results of this study and those of previous works (Minnich, 1921, 1922a, 1922b; Anderson, 1932; Weis, 1930; Frings and Frings, 1949) indicate that the butterflies (Superfamily: Papilionoidea) generally have contact chemoreceptors sensitive to sucrose and mediating feeding responses (trophic contact chemoreceptors) on the tarsi and distal part of the proboscis. The following species have been shown experimentally to possess them:

Family: Satyridae
Lethe eurydice
Cercyonis pegala
Family: Danaidae
Danaus plexippus
Family: Nymphalidae
Speyeria cybele
Limenitis arthemis
Family: Pieridae
Colias philodice
Pieris rapae

The following species have been shown to have contact chemoreceptors on the tarsi and almost certainly on the proboscis, but the latter has not yet been shown experimentally.

Family: Lycaenidae
Lycaena thoe
Family: Nymphalidae
Phyciodes tharos
Nymphalis antiopa
Vanessa atalanta
Limenitis archippus
Family: Papilionidae
Papilio machaon
Papilio polyxenes

In the Superfamily Hesperioidea (Skippers), *Polites mystic* has been reported by Anderson (1932) as showing only questionable discrimination by the tarsi between sucrose solutions and water. The proboscis was not tested. From preliminary observations on eight individuals (*Hesperia* or *Polites* spp.) it seems highly probable to us that tarsal and proboscidal receptors are present, but more

critical tests are needed. These Lepidoptera, as noted by Anderson, are erratic in response, often going for three or four days without taking water or sucrose solutions.

Among moths, Weis (1930) has reported that the following species lack tarsal chemoreceptors; other parts were untested.

Family: Sphingidae
Macroglossa stellatarum
Acherontia atropos
Sphinx pinastri
Family: Saturniidae
Aglia tau

Our results show that the following have tarsal, proboscidal and antennal receptors.

Family: Amatidae
Ctenucha virginica
Scepsis fulvicollis

The situation in moths needs much more study with many more species from a number of families. Many species of moths are known to have rudimentary mouthparts and to pass the adult life without feeding; it seems hardly likely that they would have contact chemoreceptors. The habits of the sphingid moths would seem to limit the utility of tarsal chemoreceptors. The day-flying, flower-feeding habits of the Amatidae make their sensory requirements like those of butterflies. A broader study might indicate how far this seeming adaptation of chemoreceptors to habits is found.

The results with the antennae of *Scepsis* and *Ctenucha* clearly mean that further testing is necessary before it is safe to conclude that antennal receptors are not present in other Lepidoptera. It is obvious that the conditions under which tests of contact chemoreception are made must be carefully specified. It seemed earlier (Frings and Frings, 1949) that antennal contact chemoreceptors were present only in Hymenoptera. Since then, Roth and Willis (1952) have reported them in cockroaches. They used sexual reactions rather than feeding reactions. The change in behavioral index of reception thus changed the interpretation of the location of the receptors. With the legs present, *Scepsis* and *Ctenucha* seemed to lack antennal trophic contact chemoreceptors. With the legs removed, they seemed to be present. With 1 *M* sucrose solution, *Scepsis* seemed to have receptors only on the distal segments of the mesothoracic and metathoracic tarsi. But with saturated sucrose solution, all segments were clearly sensitive. Regardless of theoretical interpretations one wishes to make of these cases, the necessity for exact specification of conditions of testing is obvious. All the receptors available to the animals may not be used in any specific behavioral situation (Tinbergen, 1951, pp. 25–37), or the receptors used in a particular behavior pattern may have different thresholds. Further, we may conclude that, while positive reactions in tests such as these indicate the presence of contact chemoreceptors, negative reactions do not necessarily show their absence. Negative results must be interpreted with great caution.

SUMMARY

1. The loci of contact chemoreceptors, stimulation of which by sucrose solutions elicits proboscis extension, were determined for the butterflies: *Cercyonis pegala* and *Lethe eurydice* of the Satyridae, and *Speyeria cybele* and *Limenitis arthemis* of the Nymphalidae; and the diurnal moths: *Ctenucha virginica* and *Scepsis fulvicollis* of the Amatidae.

2. Local contact and excision methods were used. All have the receptors on the terminal part of the proboscis and on all the functional tarsi. The palpi probably do not bear the receptors, and in the butterflies the antennae likewise. In *Ctenucha* removal of the first two pairs of legs and in *Scepsis* removal of all the legs resulted in feeding reactions on stimulation of the antennae with sucrose solutions.

3. These results emphasize the need for careful control and specification of testing conditions and for cautious interpretation of negative results in experiments such as these.

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ON REGULATION OF CONCENTRATION AND CONTENT OF CHLORIDE IN GOLDFISH

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Only little is known about the variations which may normally occur in the content of water and ions of fresh water fishes. Such variations are determined by the rates at which water and ions are taken up or lost. Water is continuously being absorbed through the outer surfaces, various ions may be taken up actively by the gills or ingested with the food. Loss takes place through the kidneys and the surface. The renal loss varies with the urine flow and ionic concentration of the urine which is generally very dilute. Loss of ions by diffusion through the skin also varies. It is for instance strongly increased in disturbed or wounded fish (Krogh, 1937; Meyer, 1948).

The capacity for uptake of sodium and chloride ions from dilute solutions is highly developed in many fresh water fishes (Krogh, 1937, 1939; Meyer, 1948, 1951; Mullins, 1950; and Wikgren, 1953). In the stickleback *Gasterosteus aculeatus* Mullins showed that one quarter of the amount of sodium in the body is exchanged per day. The osmotic and ionic concentration is, however, kept rather constant despite a high rate of turn-over of ions. The osmotic pressure is about 300 m.osM./l in fresh water teleosts (Duval, 1925; Krogh, 1939; Vinogradov, 1953; Robertson, 1954). Variations in content of sodium and chloride ions—if they occur to any degree—are therefore presumably accompanied by such variations in water content that the osmotic pressure of the body fluid remains constant.

In the present investigation we followed during several weeks the daily changes in chloride content and weight of undisturbed starving goldfish. From these data it was possible to estimate daily and long-term changes in osmotic pressure and in total content of water and chloride of the body. The chloride content of the body was also measured by the dilution method using the radioactive isotope Cl^{36}. Changes in chloride content could thus be compared with the total amount of exchangeable chloride in the fish.

Technique

Healthy, 10–20 g., goldfish were used. They were kept in aerated chambers of the type previously described (Jørgensen, Levi and Ussing, 1946). The experiments were performed during the months of March to May in an unheated room where the temperature varied from 15 to 20° C.

Daily variations in chloride content of the fish were calculated from changes in chloride content of the medium, which was 200 ml. tap water, of initial concentration about 1–2 m.eq./1. Chloride was determined in one-ml. samples according to Rehberg in Schnohr's (1934) modification. The chloride content of the medium was estimated with an accuracy of about 5 μeq. Only changes amounting to some 10 μeq. and more in chloride content are therefore significant.

Great care was taken not to disturb the fish unduly during the weighings, because handling itself can induce loss of weight and of chloride. The fish, which apparently got accustomed to the weighing procedure, were carefully transferred from their chambers into nets which were drained on filter paper. From the nets the fish were put into weighing-glasses without being touched directly by the hands. Weighings were made in duplicate. The fish were returned to water between the two weighings which were thus performed under the same conditions. The weight in grams was read with two decimals and rounded to 0 or 5. The duplicates deviated maximally by 100 mg. Daily changes in weight should therefore amount to 0.2 g. or more in order to be significant.

Total exchangeable chloride was determined in three well-nourished goldfish which were kept in 200 ml. tap water enriched with Cl^{36}-labelled sodium chloride. Changes in specific activity of the chloride in the medium were followed. From the

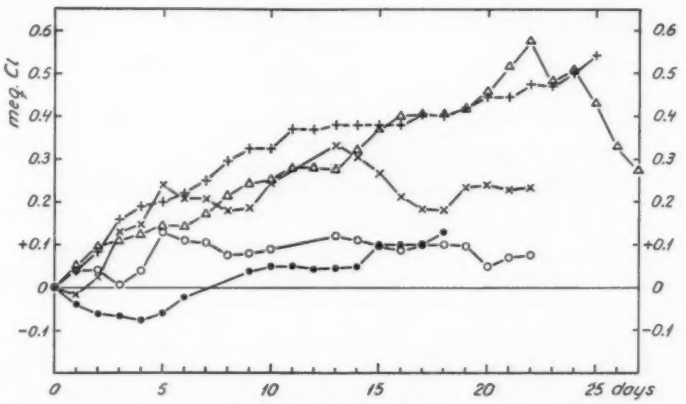

FIGURE 1. Spontaneous changes in chloride content of 5 starving goldfish, initially weighing 11–19 g.

degree of dilution of Cl^{36} the amount of chloride in medium plus goldfish was computed. Radioactivity was measured on evaporated 0.5-ml. samples of the medium by means of a Geiger-Müller end-window counter.

RESULTS

Variations in chloride content were followed during several weeks in five goldfish. As may be seen from Figure 1, uptake or loss of chloride often continued for several days or even weeks at a time, resulting in large changes in total chloride content of the body. One fish, weighing 19 g., took up 600 μeq. of chloride in 27 days, and another one, weighing 17 g., took up 575 μeq. in 22 days. Total exchangeable chloride, determined in three fed goldfish, was found to be 34.5, 35.5 and 43.0 μeq./g. body weight. Assuming an initial chloride content of 38 μeq./g., the two above mentioned fish thus increased their chloride content by 83 and 89 per cent, respectively.

Even the day-to-day changes in chloride content can be considerable. All avail-

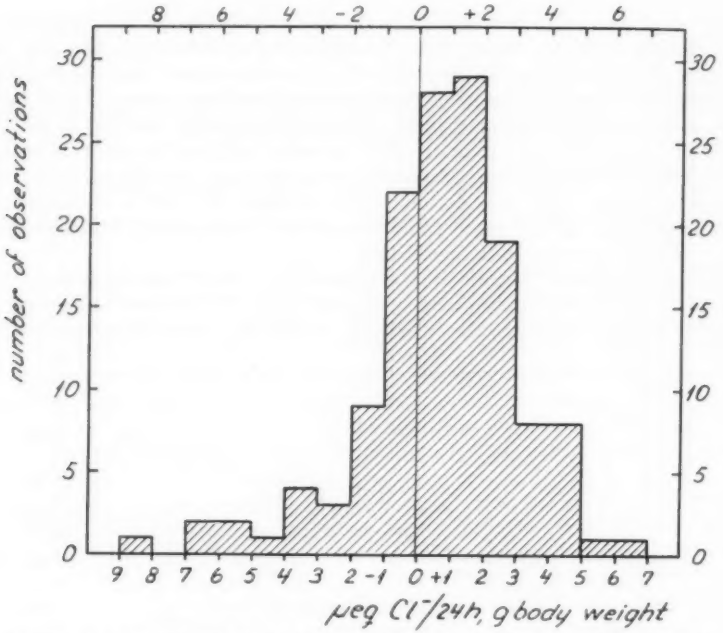

FIGURE 2. Spontaneous daily changes in chloride content of 9 starving goldfish.

able observations, 138 in number, on spontaneous daily changes in chloride content of nine goldfish are given in Figure 2, expressed in μeq. per gram of body weight. Maximum daily gains of 6–7 μeq. per gram correspond to $6.5/38 = 17$ per cent change in chloride content and maximum loss of 8–9 μeq. to some 22 per cent. Without proportional change in water content such large variations in chloride content would result in a strongly varying osmotic pressure. The daily changes in weight were therefore measured simultaneously with the daily changes in chloride content in order to estimate how constantly the osmotic pressure was maintained. The animals were adapted to the experimental conditions for ten days prior to the

TABLE I

Regression of daily weight changes (g.) of starving goldfish on daily changes in chloride content (m.eq.)

Experiment No.	Regression equations	Correlation coefficients	Statistical significances	Maximal daily changes in Cl content (μeq.)
1	$y=6.3x-104$	0.719	$0.02>P>0.01$	−45+23
2	$y=6.7x-112$	0.762	$0.01>P>0.001$	−53+54
3	$y=4.2x-97$	0.645	$0.05>P>0.02$	−46+41
4	$y=1.4x-108$	0.320	$0.3>P>0.2$	−9+46
5	$y=5.1x-216$	0.867	$P<0.001$	−87+64

measurements which were extended over a period of 11–15 days. A statistically significant correlation was found between daily chloride and weight changes in four of the five goldfish under experiment (Table I). In experiment 4 without significant correlation the daily variations were small. It may be seen from the table that the larger the daily changes the more significant was the correlation.

The regression coefficients in experiments 1, 2, 3 and 5 are 4.2, 5.1, 6.3 and 6.7. The mean value is 5.6. Thus, a change in chloride content of one m.eq. was accompanied by a change in weight of 4.2 to 6.7 g. Or in other words retention or excretion of 150 to 238 μeq. Cl was followed by retention or excretion of one ml. water.

The regression equations further show that in the five goldfish a chloride-independent loss of weight took place, ranging from 97 to 216 mg. daily. Computed per 100 g. body weight the values are 0.64, 0.75, 0.87, 0.88 and 1.34 g. which probably mainly correspond to the daily averages of break-down of tissue due to the metabolism.

Discussion

In the goldfish and other fresh water fish, chloride can be taken up through the gills and ingested with the food or by drinking. It might be of interest to estimate the relative importance of these sources. From Figure 2 it can be seen that in some 25 per cent of the periods of observation from 200 to 700 μeq. Cl was gained per day and 100 g. body weight. The metabolic rate was measured in three of the fish under experiment. Oxygen was determined by means of the Winkler technique. Measurements of the oxygen consumption were performed on undisturbed fish which were not removed from their experimental chambers; 0.233, 0.240 and 0.237 l. of oxygen was consumed per 24 h. and 100 g., corresponding to a combustion of about ¼ g. mixed organic matter. Even when the metabolic rate of starving fish is lower than normal probably less than one gram of organic matter is needed daily in order to cover the energy requirements. If one gram were to be obtained in the form of animal food containing, say, 70 per cent water and 40 μeq. Cl/g., this would correspond to an ingested amount of 130 μeq. Cl per 24 h. and 100 g., or only a quarter of an amount which is easily taken up through the gills. Presumably much less than 40 μeq. Cl/g. is contained in the food of the goldfish which eat much plant material, partly decaying, and poor in chloride.

Recent investigations of Allee and Frank (1948, 1950) show that some drinking takes place in fresh water fishes. Chloride uptake by drinking can, however, only be negligible in the goldfish. The daily diuresis amounts to some 10 ml. per 100 g. Most of this water is probably taken up through the outer surface, especially the gills (Krogh, 1939). Thus, at most a few μeq. Cl can be obtained by drinking in ordinary fresh water which generally contains less than one m.eq. Cl per liter. Under normal conditions, therefore, the gills must be more important organs for absorption of chloride than is the alimentary tract.

The total amount of chloride in the body is not precisely regulated in the goldfish, at least not in starving animals, whose chloride content could be almost doubled. The chloride concentration and osmotic pressure are better regulated. A loss or gain of only some 0.3 m.eq. Cl/100 g. within 24 hours generally coincides with a corresponding loss or gain of water (Table I). An increase in chloride

content of 0.3 m.eq. per 100 g. body weight would produce an increase in osmotic pressure of about 3 per cent if no compensatory changes took place, assuming body water to be 70 per cent of the body weight and osmotic pressure to be equivalent to 300 m.osM./1. The osmoregulatory mechanisms are therefore probably sensitive to variations smaller than ±3 per cent of the osmotic pressure when such change occurs in 24 hours or less.

It was found that loss or gain of 150 to 238 μeq. Cl corresponded to a change in body water of one ml. However, the chloride concentration of the body fluid is lower than 150 m.eq./1, probably about 117 m.eq. as found by Robertson (1954) in the powan *Coregonus clupeoides*. Periods of steady uptake of chloride must consequently result in increasing concentrations of chloride and presumably also osmotic pressure of the body fluid. Periods of loss, on the other hand, must ultimately lead to decreasing chloride and osmotic concentrations. It thus seems as if the levels at which chloride and osmotic concentrations are maintained vary with the total chloride content of the body. It can further be assumed that the increase in chloride or osmotic concentrations which follows chloride uptake stimulates mechanisms that eventually result in net loss of chloride. Similarly, decreases in chloride or osmotic concentrations may be supposed finally to stimulate uptake and to reduce excretion of chloride, so that body chloride is once more increasing or perhaps kept constant for some time. Volume and osmotic regulation thus seem to be mutually dependent within a system that permits much larger variations in chloride content than in chloride concentration and consequently also osmotic pressure.

Summary

Daily changes in chloride content and weight of undisturbed starving goldfish were followed during several weeks. Uptake or loss of chloride often continued for several days or even weeks at the time. Up to 83 and 89 per cent increases in chloride content were measured. Maximum daily gains were 17 per cent, and losses 22 per cent of total chloride content. A statistically significant correlation was found between daily chloride and weight changes. Retention or excretion of 150 to 238 μeq. Cl was followed by retention or excretion of one ml. water.

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RELATIVE EFFECTIVENESS OF DEHYDRATION AND NEUROHYPOPHYSIAL EXTRACTS IN ENHANCING WATER ABSORPTION IN TOADS AND FROGS

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It is well known that the vertebrate neurohypohysis contains substances which can increase the permeability to water of the skin of amphibians and reduce the rate of urine flow. The same effects can be produced by dehydration or by injection of hypertonic solutions. It has, therefore, been suggested that these treatments act by stimulating the neurohypophysis to liberate so-called water balance hormone. Ultimately, neurohypophysial activity should thus be responsible for at least part of the renal and cutaneous effects of hydropenia in amphibians (Eliassen and Jørgensen, 1951; Ewer, 1952; Jørgensen, 1950; Sawyer and Sawyer, 1952). It thus seemed of interest to compare the relative effectiveness of dehydration and neurohypophysial extracts in enhancing water uptake. Experiments were made on the toad *Bufo bufo* (L.) and the frog *Rana temporaria* (L.).

Technique

Water uptake was measured as previously described (Jørgensen, 1949). The animals were placed in stainless steel wire cages and immersed in tap water. The rate of water absorption was determined by weighing animal plus cage at suitable intervals, generally one or one half hour. The animals were dehydrated in glass beakers covered by gauze, at a rate corresponding to a daily weight loss of about 10 per cent. Injections were given subcutaneously. Both mammalian and amphibian neurohypophysial extracts were used. Insipidin AB and Pitupartin AB served as mammalian preparations. Insipidin contains 20 international units of the pressor-antidiuretic principle per ml., Pitupartin 10 units of the oxytocic principle per ml. Insipidin has been found to be more effective than Pitupartin in enhancing water uptake in the toad. In the experiments on toads Insipidin was consequently used, 2–5 U being injected per 100 g. body weight to ensure maximum effect on water uptake (Jørgensen, 1950). In frogs the oxytocic principle is apparently more effective than the pressor-antidiuretic principle of commercial neurohypophysial preparations (Heller, 1945). In our experiments maximal rates of water uptake were induced by injection of about 5 U of Pitupartin per 100 g. frog. Extracts of the neurohypophyses of frogs and toads were made either in the usual way by extracting the minced glands for about 5 minutes with boiling 0.25 per cent acetic acid or by finely grinding the glands in a mortar with a little sand and a drop of Ringer solution. The comminuted tissue was suspended in Ringer solution and injected. Maximal stimulation of water uptake was obtained with amounts of ex-

tract corresponding to less than one-tenth of a gland from an animal of the same species and of the same size as that into which the extract was injected. In the present experiments extract from one-tenth of a toad gland was injected into each toad and one-fifth of a frog gland into each frog.

Results

The effect of hydropenia on the ability of toads and frogs to take up water through the skin varies with the temperature, the season of the year and other factors which have not been investigated systematically. An example of the large seasonal variations that can be found in the effect of dehydration on the rate of water absorption is given in Figure 1. Water uptake of normally hydrated toads

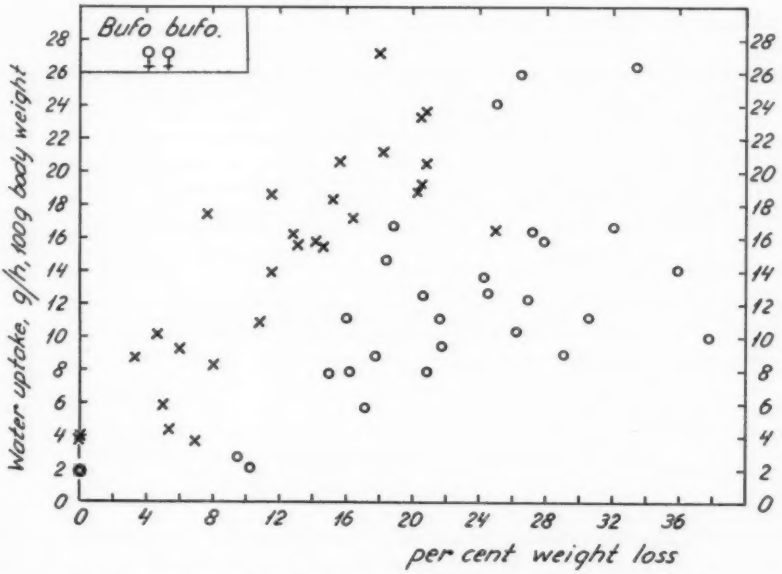

FIGURE 1. Water uptake in dehydrated female toads placed in tap water. × June; ⊙ December and March. Temp. 23–24° C.

was 4 ml./hr./100 g. in June and 2 ml. in December and March at 23–24° C. In June a weight loss of some few per cent strongly increased the water uptake, whereas in December or March a weight loss of 8–10 per cent was needed in order to affect the permeability to water. The maximal rates of water absorption, too, have been observed to be higher in summer-toads than in winter-toads. Comparisons between the effect of dehydration and neurohypophysial extracts should therefore be made at the same time of the year. Table I collates the results of a number of such comparisons between the relative effectiveness of strong dehydration and neurohypophysial extracts in enhancing water absorption in toads and frogs.

In experiments 2 and 3 of the Table the same animals were subjected to both dehydration and to injections. In experiment 1 the two types of treatment were given to separate groups of toads that had, however, been treated uniformly up to the time of the experiments. Desiccation corresponding to 20–30 per cent weight loss was much more effective than injection of supramaximal doses of neurohypophysial extracts in all experiments made at the same time of the year. There was no difference between the effect of mammalian and amphibian extracts. The pituitary extracts, however, produced larger effects during summer than during winter. In May–June toads responded as strongly to the extracts as they did to dehydration in November–December. No difference was found in the responses of the two sexes.

The increase in sensitivity to water loss and neurohypophysial extracts coincides with the onset of sexual activity in the spring. In toads imported from Italy in

TABLE I

Effect of dehydration and injection of neurohypophysial extracts on the rate of water uptake in toads and frogs. Water uptakes are expressed in ml. per hour and 100 g. body weight. The figures represent means of 5–15 independent determinations. Ranges are given in brackets

No. of experiment	Species	Time of year	Temp. °C.	Sex	Normally hydrated	Dehydration corresponding to 20–30% weight loss	After administration of:		
							Ringer's solution	Insipidin 2–5 U/100 g.	1/10 toad neuro-hypophysis
1	*Bufo bufo*	Nov. Dec.	17–19	♀	1.1 (0.8–1.7)	7.5 (5.1– 9.5)	1.3 (0.8–2.3)	2.0 (1.1– 3.3)	1.9 (1.1–2.9)
2	*Bufo bufo*	May	21–22	♀	1.7 (1.1–3.5)	18.7 (12.9–34.8)	2.0 (1.5–2.9)	8.1 (5.3–10.7)	
		June		♂	1.8 (0.9–3.6)	20.9 (9.0–38.8)	2.3 (1.4–2.8)	7.4 (4.4–11.3)	
								Pitupartin 5 U/100 g.	1/5 frog neuro-hypophysis
3	*Rana temporaria*	May	23–25	♀ ♂	2.4 (1.6–3.9)	19.6 (16.3–22.1)		4.8 (2.3–6.5)	5.3 (2.5–7.8)

February during the breeding period, 17–24 per cent weight loss resulted in water uptakes of 16–21 ml./hr./100 g. at 18–19° C. Extracts of 1/10 of a toad-neurohypophysis increased water absorption to the high values of 7–13 ml. At the same time Danish toads were still sexually inactive and showed the typical low "winter-response." The strong water balance reaction persists beyond the breeding period as may be seen from experiment 2 of the Table, performed one–two months after reproduction had taken place.

DISCUSSION

Dehydration was found always to be more effective than injection of neurohypophysial extracts in increasing the rate of water uptake through the skin of toads and frogs. If, therefore, enhanced water absorption after desiccation is produced by water balance hormone liberated from the neurohypophysis it follows that injected water balance principles must be less effective than hormone liberated

from the animal's own neurohypophysis. However, in man and dog Pitressin can produce a maximally concentrated urine and thus be as effective as naturally liberated antidiuretic hormone (O'Connor, 1950; West, Traeger and Kaplan, 1954). More likely, therefore, the effect of hydropenia on the permeability of the skin of toads and frogs is not exclusively due to increased neurohypophysial activity. At strong dehydrations the neurohypophysis is presumably of only minor importance in enhancing the ability to absorb water.

Summary

Comparisons have been made between the effect of strong dehydration (20–30 per cent weight loss) and of supramaximal doses of neurohypophysial extracts on the rate of water uptake through the skin of the toad *Bufo bufo* (L.) and the frog *Rana temporaria* (L.). Hydropenia increased the permeability to water much more than did injection of the pituitary extracts. Extracts of mammalian and amphibian glands were equally effective. The responses to desiccation and to injection of neurohypophysial extracts were larger during summer than during winter.

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ON PIGMENT CHANGES AND GROWTH IN THE BLUE-GREEN ALGA, PLECTONEMA NOSTOCORUM BORNET EX GOMONT

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Changes in pigmentation in the algae, as influenced by environmental conditions, have evoked considerable interest in the past and at present. It is the purpose of this paper to describe one of the lesser known instances in which a specific change in pigmentation follows a specific change in the environment of an alga, and some of the practical considerations that may be drawn therefrom.

The theory of chromatic adaptation in the algae commenced in the late 19th century with the observation that there was a relationship between color and depth of zone of growth of marine algae, and has had a controversial course since. Rabinowitch (1945) and Boresch (1932) may be consulted for an extended discussion. Several points have gradually become apparent.

One of the lesser publicized of these has been the observation that in certain blue-green algae, color may vary with nutrition in a dramatic fashion. Apparently the first such observation was made by Boresch (1910) with an unidentified member of the Oscillatoriaceae. He found that after a protracted period of culture, the alga lost its characteristic blue-green color and took on the light brown to golden color of a diatom. Experimentation showed that it was not the wave-length of light, but rather the depletion of nitrogen from the medium, and that alone, which was responsible for the color shift. In this and further work (Boresch, 1913; Magnus and Schindler, 1912; Schindler, 1913), all with members of the Oscillatoriaceae and especially species of *Oscillatoria* and *Phormidium*, it was determined that the color shift was due to a selective disappearance of chlorophyll and phycocyanin, thereby "unmasking" the carotenoids; that a yellowed culture could be made to return to green, sometimes within 24 hours, upon the addition of minute amounts of nitrogen [as little as "1/20,000 normal" KNO_3 (Boresch, 1910) (0.00425 grams per liter)] as nitrate or ammonium ions or various organic nitrogen sources; and that greening would take place in the absence of light. This work was done in the period of active debate concerning the validity of theories of chromatic adaptation. Light was indirectly involved in these experiments since the greater the availability of light, the sooner the nitrogen content of the medium would be used up, other factors being equal, and the quicker and more markedly the color shift would take place. It was noted that the chromatic adaptation, and color change due to nutrition concepts as related to light were ecologically antipathetic. The former states that color shifts occur to allow the organism to adapt itself for the better use of light received, thus increasing its phototrophic assimilation, while the latter states that phototrophic assimilation is decreased by the color shift because of the disappearance of the primary photosynthetic pigments. Although the green to yellow color shift reduces or eliminates phototrophic assimilation, it was held

nevertheless beneficial to the alga in that it allows the organism to enter a state of dormancy until conditions ameliorate.

Material

In the course of other work a pure culture of a blue-green alga was obtained from a natural collection through the use of ultraviolet irradiation. This organism was identified by Dr. Francis Drouet as *Plectonema nostocorum* Bornet ex Gomont. It is somewhat unusual in its minute dimensions, being composed of sheathed trichomes of undifferentiated cells averaging 1.3 μ in width at the widest point by 1.8 μ in length. The sheath increased the width of the filament to 2.25–3.0 μ. Since no heterocysts, akinetes or other possibly resistant entities are formed by this species, and since it has no powers of morphological modification (except perhaps variation in width of the sheath), adaptive reactions to the environment are necessarily at the physiologic level.

Experimental

Nutritionally mediated color shift

Plectonema nostocorum had not been in culture long before the color shift was observed in some cultures. Cultures were grown in 250-ml. Erlenmeyer flasks in several liquid media. These were kept under conditions of controlled temperature (22° C. ± 1° C.) and light (40 foot candles, fluorescent source). Actively growing cultures are a brilliant emerald green. If they are kept in the same culture solution over an extended length of time in the light, the culture will, within the space of a few days, undergo a dramatic color shift from brilliant emerald green through yellow-green to a final yellow with slight overtones or orange or brown. In the latter condition, further lapse of time brings about a slow bleaching so that cultures that have been kept for many months assume the color and resemblance of dirty yellowish cotton-wool. Although at first it was assumed that this color shift was brought about by the death of the organism, further experimentation soon established the fact that it was, rather, based on the exhaustion of the mineral solution in which the organism was growing and that the organism even after many months in the new, yellowed condition was still viable. The addition of small amounts of nitrate to the yellow culture caused it to return to the original green at a very rapid rate. The beginnings of the color shift from yellow to green could be observed within about 24 hours and the shift became complete within three days. Several other elements added similarly had no such effect.

Spectrophotometric analyses of growing and dormant (yellow) cultures have been made. In preparation for this examination, living material was suspended in a few milliliters of culture solution and broken up to as small filament fragments as possible in a special small plastic mortar. The resulting suspension was diluted with distilled water to a degree necessary to give an optical density of approximately 0.65 at the wave-length of greatest absorption. The spectrophotometer used to measure the absorption at the various wave-lengths was a Coleman Universal, Model 14, following the procedures suggested by the manufacturer.

The spectral curve so obtained is somewhat flattened by turbidity and cell mate-

rial other than pigments in the sample. It was not possible to break up the filaments entirely, and the resulting sample showed a range of particle size from just visible to the naked eye down to fragments of filaments one or two cells long. The measurements were checked at wave-length intervals of every 100 millimicrons for possible changes in the reading due to settling of the sample. It was found that the greatest effect was a change of only 0.01 units of optical density over the time of the entire assay.

Although no attempt has been made to determine the exact identity of the pigments contained in the cells of this alga, the absorption curve is reproduced (Fig. 1)

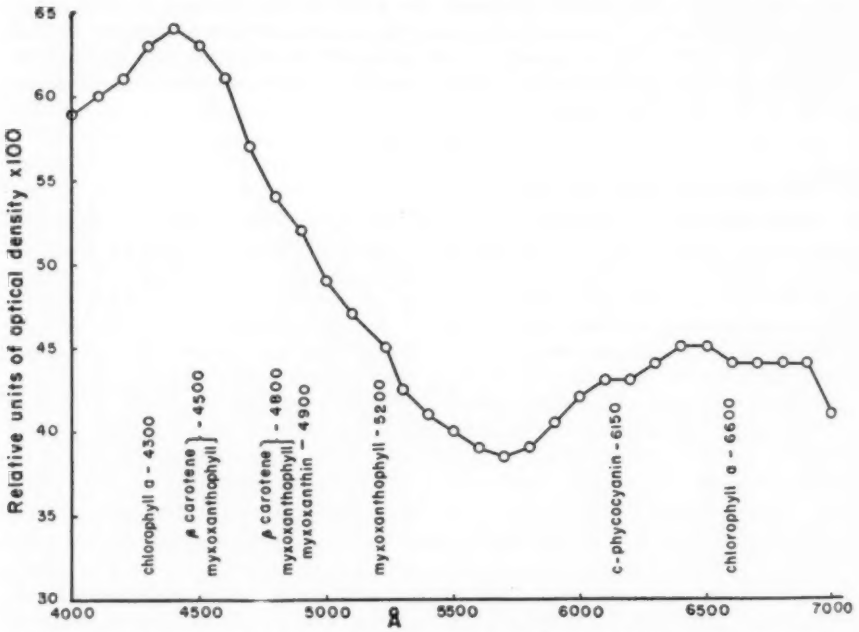

FIGURE 1. Spectrographic analysis of a green, growing culture of *Plectonema nostocorum*.

together with the approximate maximum absorption values for the pigments known to occur in the Cyanophyta (Boresch, 1932; Cook, 1945; Strain, 1951). It will be seen that there is good correspondance between the maxima of pigments generally found in the Cyanophyta and the general shape of the absorption curve for this alga in the active state, except in the region around 640 and 650 millimicrons. Chlorophyll b and c-phycoerythrin are apparently absent in this organism.

Spectrophotometric analyses of the living but yellowed pure cultures were made to determine what pigments had disappeared. As shown in Figure 2, both the chlorophyll and phycocyanin peaks are missing, the major absorption of the cultures now being represented by the presence of carotenoids only.

Growth estimation by pigment analysis

In further exploratory work with this organism it was necessary to develop an accurate method of measuring growth. Because of the small size, filamentous nature, and slow rate of growth of the organism, several of the more usual methods of measuring growth were found to be inadequate. Attention was then given to measurement of growth by means of pigment extraction and its quantitative determination.

Growth is an elusive concept and while it is recognized that pigment measurements do not necessarily precisely indicate the amount of organic matter fixed in the organism because of superimposed ecological variations, on the other hand, if

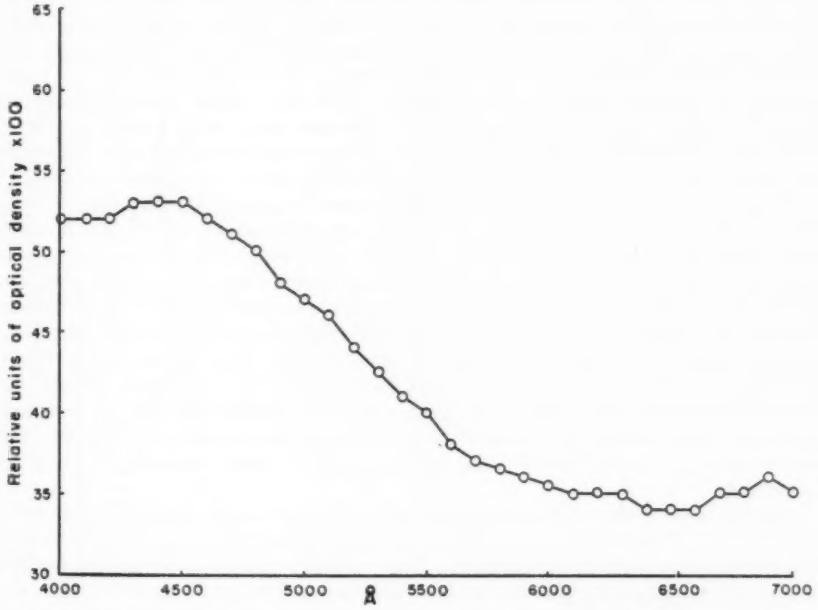

FIGURE 2. Spectrographic analysis of a yellowed culture of *Plectonema nostocorum*.

one allows a more dynamic concept of growth such as total current organic matter-producing potential, then pigment measurement assumes a greater significance (Harvey, 1950). Careful pigment analysis can also be used to characterize and estimate plankton populations (Richards, 1952).

The first to use a quantitative pigment measurement for determination of growth was Harvey (1934) who extracted (with 80% acetone) the pigment from marine plankton collected by net from a known volume of water, and compared this extract visually with calibrated dilutions of a colored standard. This method proved satisfactory for Harvey's work which was with natural populations consisting largely of diatoms. Later Riley (1938) attempted to apply the method to the

measurement of fresh water plankton populations but found that these had a too great and too variable carotenoid content. He was able to adapt the method to his needs by first extracting the chlorophyll from the total pigment complement and measuring it alone visually against calibrated standards. Gardiner (1943) determined, however, through microscopic examination of extracted sample residue, that under these conditions, not all species give up their pigments to the solvent. Further critical work was done by Tucker (1949) who compared results as determined by Harvey's method with actual counts. He found that the correlation in natural populations was not close, and it was his conclusion that it is impossible to get a fixed plankton count as equal to a particular pigment density. This is corroborated in more recent work by Berardi and Tonolli (1953) and Ramamurthy (1953). This might have been expected because of the diverse nature of plankton, containing, as it does, representatives of a wide range of plant groups and an even wider range of animal life, its relative composition depending upon numerous factors such as season, latitude, mineral composition of the waters and others. Comparisons of pigment analyses after Harvey with dry weight determinations by Margalef (1954) have also given a poor correlation even when uni-algal cultures were used. Variation in dry weight per one Harvey and correlated unit of pigment was greater than one power of ten with variations in several factors of culture.

Since none of the above listed difficulties ensues if pigment extractions are made from a pure culture of a pigment-producing organism which has been cultivated under carefully controlled conditions, it might be assumed that the amount of pigment so extracted, measured photoelectrically, would correlate closely with the amount of growth as measured by any other reliable method, especially if the chosen pigment was closely associated with the basic physiology of the cell as are the photosynthetic pigments in autotrophic organisms. This has been borne out in at least one instance. Tucker (1949) found that the coefficient of correlation between the number of cells per unit volume in a pure culture of *Ankistrodesmus*, as measured at various times during the course of its growth, and the amount of chlorophyll per unit volume as sampled at the same times, was 9.97 which represents very close agreement.

On the basis of the above information an attempt was made to establish a workable method of determining growth of the alga through the use of photoelectric measurements of pigment extractions. The immediate problem developing was the decision of which pigment to extract and measure or whether it would be more desirable to measure all pigments at once. Because, as previously described, when the nitrogen composition of the medium becomes sufficiently reduced, the alga loses its green pigments and phycobilins and turns yellow, any method to be fully satisfactory should allow the quantity of growth to be accurately measured whether the culture is in the yellow or green condition. Thus extraction must be done in such a way as to obtain only the yellow pigments which are always present during the growth of the alga and remain after growth ceases.

Extractions of cultures in both the green and yellow conditions were made with various solvents such as acetone, petroleum ether, and methyl alcohol and the extracts were analyzed for pigment complement with the Coleman spectrophotometer as previously described. The effect of the use of heat to destroy enzyme activity quickly and thus prevent degenerative pigment changes was also investigated as

opposed to extractions without this previous treatment. It was found that extractions of green cultures without heat and with 80% aqueous acetone yielded the same pigments as were obtained from yellow cultures treated similarly. The phycobilins are water-soluble and are not extracted from the cells by this method. On the other hand the chlorophyll is apparently destroyed in the extraction. It is quite apparent to the eye that as the acetone is added to the culture, there is a definite and rapid yellowing both of the solution and the filaments themselves. Although acetone solution is a normal procedure for the successful extraction of chlorophyll, it is probable that in this case the chlorophyll is destroyed by reason of the pH at

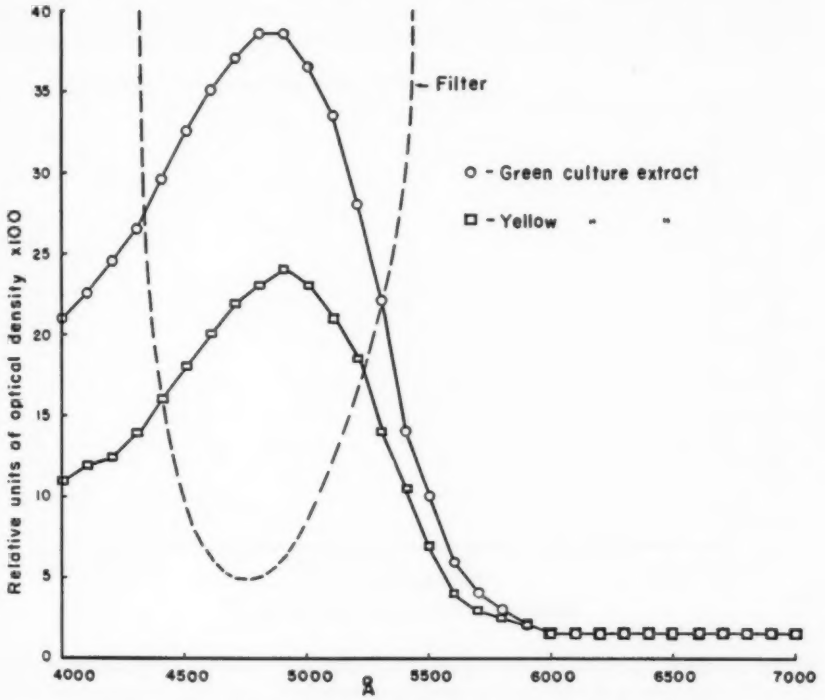

FIGURE 3. Spectrographic analysis of acetone extracts of green and yellow cultures of *Plectonema nostocorum*.

which the extraction is done. Chlorophyll is stable only near neutrality or at a slightly acid reaction. Extraction in this instance is from a culture which has been grown at a pH of 10. Although the culture solution is removed before extraction, the filaments presumedly remain basic. In any case, that the chlorophyll is destroyed and does not appear in the solvent may be seen in the spectrographic photometric analysis of the latter. Figure 3 represents such an analysis in which the curves for 80% acetone extractions of both green and yellow cultures are

plotted. It will be seen that the absorption curves of these are similar, and it is presumed that the pigments are the same. The fact that the absolute amount of pigment is different is not significant since not only were the cultures grown under different conditions for different lengths of time, but also different dilution factors were used in their preparation for analysis.

It should be possible, therefore, to determine the amount of growth in a culture, whether the latter be yellow or green, through a photoelectric determination of the amount of pigment contained in an 80% acetone extract of the culture, other conditions being controlled and equal. A filter was obtained, for use in the Klett-Summerson colorimeter, whose transmittance characteristics resembled the absorption curve of the pigment extract. The transmittance curve for the filter, Wrattan (Eastman Kodak Company) gelatin filter number 45, is reproduced in Figure 3.

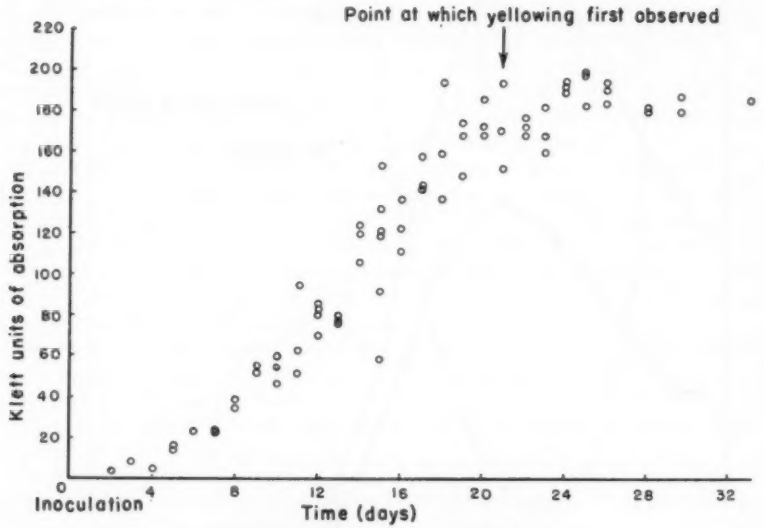

FIGURE 4. Plot of raw growth data.

Three of these filters in series equaled the transmittance of a glass filter made expressly for use in the Klett-Summerson instrument. It was further determined that the heat developed at the surface of the filter was not great enough to damage the gelatin, at least over the length of a normal pigment analysis.

A series of 250-ml. Erlenmeyer flasks was prepared. The nutrient solution was made up with CP grade reagents according to Chu's No. 10 formula (Chu, 1942) in glass-redistilled water and Chu's micrometabolic elements (Chu, 1942) made up similarly were added. The pH was adjusted to 9.7. One hundred ml. of nutrient solution were placed in each of the flasks and autoclaved. Inoculation of all flasks was from the same culture at the same time. One milliliter of this culture, which had been homogenized as much as possible in the Waring Blendor, and which had previously been tested for purity from contamination by inoculation into meat extract

broth, was aseptically introduced into each of the flasks. These were then incubated under constant conditions of light and temperature.

Starting two days from the date of inoculation, one or more cultures were removed daily and assayed in the following manner. The culture solution was filtered through a No. 1, 4.25-cm. Whatman filter disc. The filter paper was then placed in the flask, and the algal material was extracted with 80% aqueous acetone for one hour. The extract was then filtered into a test tube and compared with an 80% acetone blank for pigment absorption using the filter described above in the Klett-Summerson instrument.

The raw data are plotted in Figure 4. It should be noted that the number of cultures removed per day varied from one to six. It should also be noted that the units of absorption are arbitrary. The scale of the instrument is plotted directly in

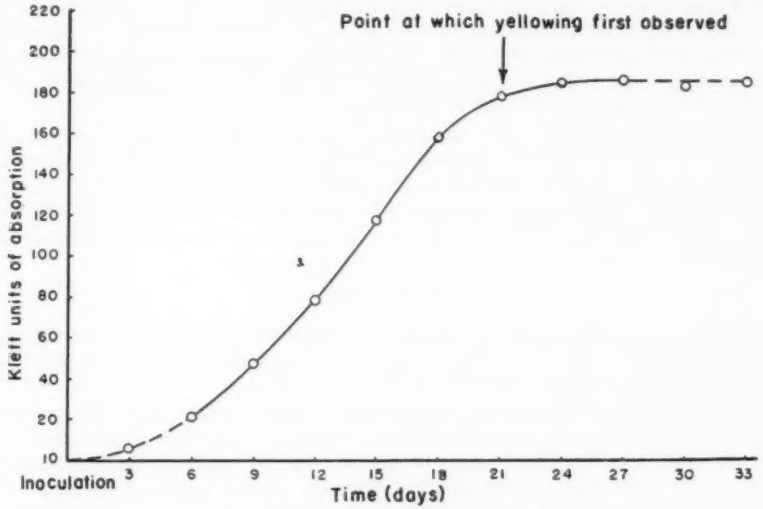

FIGURE 5. Plot of averaged growth data.

a logarithmic fashion; thus the figures are directly proportional to the concentration of the pigment being read (Lambert-Beer Law). A calibration factor, determined by reference to a known standard, may be introduced so that the final readings may be expressed in units of defined value. This was not done. Precautions were taken to insure the flasks being assayed at random and to prevent any unconscious selection on the basis of color.

The following discussion is based upon the curve reproduced in Figure 5. This curve was prepared by grouping the raw data in groups of three days, each day being given equal value, and plotting the mean of the resulting groups. It must be noted that different numbers of cultures were assayed on different days, so that there is not the same degree of statistical reliability throughout the entire curve. The greatest reliability attaches to the central part of the curve.

The point at which yellowing was first observed in the cultures by eye is noted in Figure 5. It will be seen that there is no drop in the curve at this point. It can be expected that there will be a slow decay in the curve from this point, or shortly thereafter, onward through the gradual depauperation of the culture.

As has been stated, it has proven impossible to relate the figures obtained by this method with any other criterion of growth attempted. Dry weight determinations of parallel cultures were so small as to always fall within the limits of possible experimental error until the cultures were half grown. This argues for the sensitivity of the pigment method.

Discussion

Since both chlorophyll and phycocyanin molecules contain considerable amounts of nitrogen, and since the presence or absence of nitrates from the culture medium mediates color shift, it is concluded that the alga is able, when nitrogen becomes unavailable to it from the medium, not only to cease active repairing and construction of nitrogen-containing pigments, but to remove nitrogen actively from already constituted pigment molecules, a conclusion based on the very short time in which the color shift from green to yellow takes place.

Because of the rapidity with which greening takes place in this organism, it is possible to effectively separate pigment synthesis from other growth phenomena.

In this study, pigment evaluation was used as an index of growth. Despite the considerable experimental evidence of others to the inadequacy of this procedure under different conditions, the method appears to have been successful in this instance.

Summary

1. Spectrographic analyses of cultures of *Plectonema nostocorum* are reported, which demonstrate that the nitrogen-containing pigments disappear in the living alga when nitrogen becomes depleted in the culture medium.

2. Re-greening of cultures upon the addition of nitrogen is rapid and can serve to separate the process of pigment synthesis from other processes of growth.

3. Extraction and measurement of the carotenoid pigments at any stage in growth under controlled conditions is considered a reliable index of growth.

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SKELETON FORMATION OF SEA URCHIN LARVAE. I. EFFECT OF CA CONCENTRATION OF THE MEDIUM [1]

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Although many reports are available concerning the morphological development of the sea urchin skeleton (v. Ubisch, 1913; MacBride, 1914; Mortensen, 1921, 1931; Goldon, 1926a, 1926b, 1928; Onoda, 1931, etc.), the underlying mechanism of its formation is an aspect which has not been covered by previous workers.

In marine molluscs, on the other hand, it is known that the calcium utilized in the formation of the shell is derived in part from the sea water in which the animals live. Galtsoff (1934) reported that the amount of calcium deposited to form the oyster shell was many times greater than that which could be stored in the tissues. Orton (1925) noticed in the oyster that increase in the size of the shell took place even in the absence of food. Fox and Coe (1943), in their studies on *Mytilus*, showed that the amount of calcium obtained from organic food alone was not sufficient to account for the amount deposited in the shell. In recent studies, it has been proved that an isolated "mantle-shell preparation" is able to deposit calcium from the sea water onto the shell (Hirata, 1953; Jodrey, 1953). Bevelander and Benzer (1948) have shown that there is a corresponding reduction in the calcium of the regenerating molluscan shell (*Pedalion alatum*) when the calcium content of the sea water is reduced. Further, Bevelander (1952) agrees with Robertson (1941) in the conclusion reached in Robertson's comprehensive review dealing with calcification in molluscs, that molluscs can utilize calcium in the ionic form. This conclusion is further corroborated by Rao and Goldberg (1954), who cultured *Mytilus* in filtered sea water.

In sea urchin larvae, a direct dependence on sea water for the salts or ions used for the larval skeleton is shown by the following facts: the beginning of spicule formation precedes the completion of gastrulation, hence takes place before feeding starts; spicules continue to grow in cultures which are without food; exogastrulae having no functional digestive tract are able to form typical skeletons like those of normal plutei (Okazaki, 1956). In earlier studies, it has been stated that spicules do not develop in sea water in which the calcium is seriously reduced (Pouchet and Chabry, 1889) and also that the absence of magnesium and sulphate ions arrests the spicule formation of sea urchin larvae (Herbst, 1904). However, hardly any details about skeleton formation have been clarified so far.

In the present paper, the effects of the Ca concentration of the culture medium on skeletal development will be reported as a preliminary to the study of the mechanism of skeleton formation.

[1] This research was supported in part by the Ministry of Education Research Expenditure.

Materials and Methods

The larvae of *Hemicentrotus pulcherrimus* were principally used, because the form of the skeleton of this species is the simplest to be found among the sea urchin larvae available at Misaki. As supplemental materials, *Pseudocentrotus depressus, Clypeaster japonicus* and *Mespilia globulus* were used.

Ca-low media were prepared by mixing Ca-free sea water [2] and natural sea water in 9 : 1, 8 : 2, 6 : 4, 4 : 6 and 2 : 8 ratios. As Ca-high media, 0.36 *M* $CaCl_2$ was mixed with natural sea water in 11 : 629, 11 : 309, 11 : 149, 11 : 69 and 11 : 29 ratios. The resultant Ca concentrations of the whole series of mixtures correspond to about 0.1 ×, 0.2 ×, 0.4 ×, 0.6 ×, 0.8 ×, 1.5 ×, 2.0 ×, 3.5 ×, 6.0 × and 10.0 × the calcium content of natural sea water. For convenience, these media will be referred to as 0.1 Ca, 0.2 Ca, 0.4 Ca . . . , respectively.

Larvae were cultured in Syracuse watch glasses containing about 15 cc. of medium. In these cultures, the larvae must be placed in a single layer, so as to minimize variations in the rate of development which would lead to individual differences in skeletal size. As long as the number of larvae in a container is kept below such a level, further reduction in the larval number per container does not affect the skeleton formation. This latter situation indicates that it is the concentration of calcium and not the absolute quantity of calcium available per larva which should be taken into consideration.

In all the experiments reported in this paper, the larvae were cultured in the absence of food.

Experimental Results

Before describing the effects of media with modified Ca concentrations on skeleton formation, it seems appropriate to mention their effects on the general developmental process of sea urchin larvae. These are summarized in the following five points.

(1) *Time of hatching.* In Ca-low media, no retardation of development was obvious in reference to the hatching time, while in Ca-high media, the time of hatching was more or less retarded. However, this retardation of hatching time in Ca-high media is not simply due to a generally delayed rate of development, judging from the time of mesenchyme formation and gastrular invagination, as described below.

(2) *Time of beginning of mesenchyme immigration and gastrular invagination.* With reference to the time of the onset of these activities, larvae in 0.1–3.5 Ca did not show any difference from the control, except occasional early beginning in 0.1 and 0.2 Ca. In 6.0 and 10.0 Ca larvae, however, both activities were retarded in comparison with the controls.

(3) *Gastrulation.* In 0.2–6.0 Ca, gastrulation proceeded in an almost normal manner. Larvae cultured in extreme concentrations (0.1 and 10.0 Ca) frequently developed into exogastrulae, as described in previous papers (Dan and Okazaki, 1956; Okazaki, 1956).

(4) *Size of the blastocoel.* In Ca-low media, the size of the larval blastocoel

[2] NaCl 26.5 g., KCl 0.7 g., $MgSO_4 \cdot 7H_2O$ 11.9 g., $NaHCO_3$ 0.5 g. plus distilled water to 1000 cc.

became larger than that of the control larvae. This was so particularly in 0.8 and 0.6 Ca. In Ca-high media, on the other hand, the blastocoels became smaller in proportion to the concentration of Ca present, in the same manner as was reported by Dan (1952) in this species.

(5) *Formation of coelom.* In the extreme concentrations, the secondary mesenchyme cells failed to form a typical coelom. They either formed a single vesicle on the tip of the archenteron or dispersed without coordination.

From the foregoing facts, it can be said that the concentration range of Ca which allows essentially normal development is from 0.2 to 3.5 times that of sea water.

I. Limiting concentration of calcium necessary for skeleton formation

In larvae which were cultured in even as low a concentration as 0.1 Ca from the two- or four-cell stage, doubly refractive skeletons were formed, although their form was atypical and the time of their formation was much delayed, as will be described below. If larvae reared in sea water were transferred to Ca-low sea

TABLE I

Comparison of the time of the beginning of the skeleton formation in various Ca concentrations. Numbers indicate the ratios of hours required between insemination and skeleton formation in experimental media over the control. Material: Hemicentrotus pulcherrimus

Stage of transfer \ Ca concentration	0.1	0.2	0.4	0.6	0.8	S.W.	1.5	2.0	3.5	6.0	10.0
4-cell						1.00	0.99	1.00	1.00	1.04	1.08
32-cell	2.22	1.69	1.31	1.03	1.00	1.00	1.00	1.00	1.00	1.05	1.07
Morula	2.80	1.87	1.17	1.05	1.01	1.00	1.00	0.99	1.00	1.05	1.07
Blastula before hatching	2.65	1.71	1.15	1.02	1.00	1.00	0.99	0.99	1.00	1.04	1.13
Swimming blastula with no mesenchyme cells	2.20	1.69	1.29	1.03	1.00	1.00					

water after the formation of tri-radiate spicules, skeletal growth continued for a short time even in 0.05 Ca, in which the general development was ultimately impaired. These facts show that the Ca threshold for skeleton formation is lower than that for harmonic development of the whole larva. Such being the case, the minimal calcium concentration required for skeleton formation itself is impossible to decide.

Bevelander and Benzer (1948), in their paper dealing with shell regeneration in young specimens of *Pedalion*, reported that when an operated animal was placed in sea water containing 1/8 of the normal calcium content, the organic matrix was completely devoid of calcium, although the matrix was formed in a normal manner. For sea urchin larvae, however, a medium having such an effect cannot be prepared by calcium reduction alone.

It is frequently the case that in the larvae of *Clypeaster japonicus* and *Mespilia globulus* cultured in 0.1 Ca, skeletons as such failed to appear; instead, minute doubly refractive granules were formed in the mesenchyme cells.

II. Time of the beginning of skeleton formation

Larvae fertilized and cultured in sea water were transferred to media of various calcium concentrations at various stages before the appearance of the mesenchyme

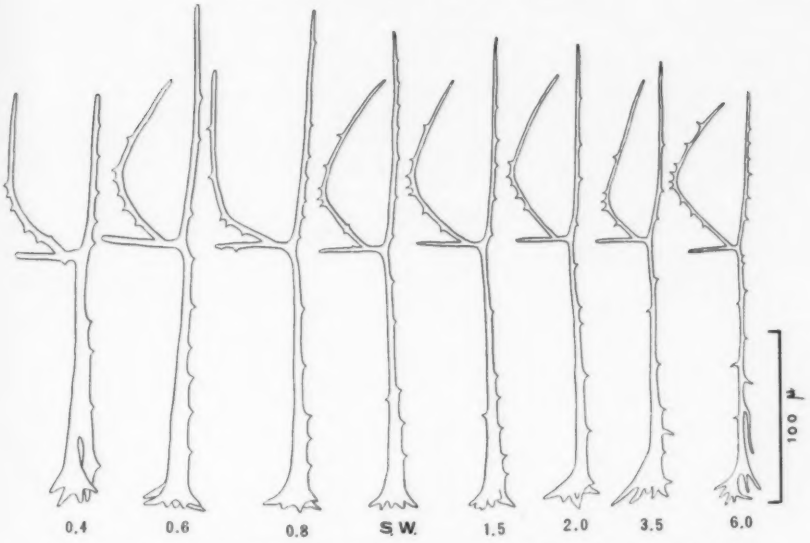

FIGURE 1. Skeletons of *H. pulcherrimus* larvae reared in media with varied Ca concentrations from 0.4 to 6.0 times that of sea water.

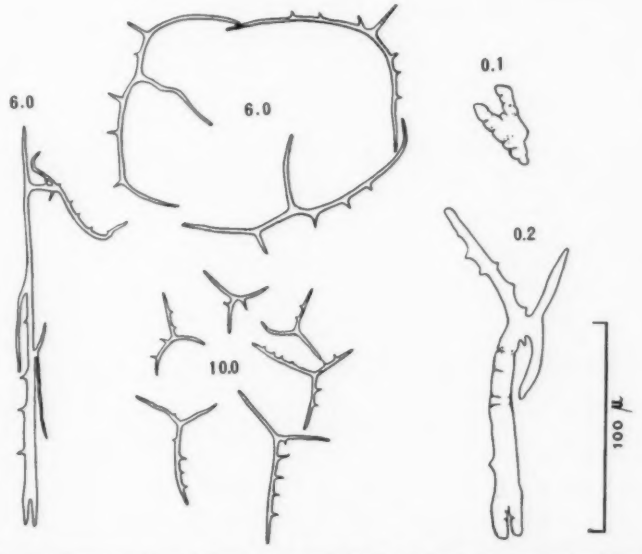

FIGURE 2. Skeletons of *H. pulcherrimus* larvae in the extreme Ca concentrations (0.1, 0.2, 6.0 and 10.0 × normal sea water.) Larvae were transferred to such media before skeleton formation.

cells, and the times of the beginning of skeleton formation were compared. As the initial stage of skeleton formation, the time was recorded when doubly refractive granules were recognized by a polarization microscope in 50% of the larvae in a given culture. The results are summarized in Table I.

As far as pre-mesenchyme blastulae were concerned, the stage at which the larvae were transferred did not affect the results. Generally speaking, in Ca-low media, the time of the beginning of skeleton formation became later in proportion

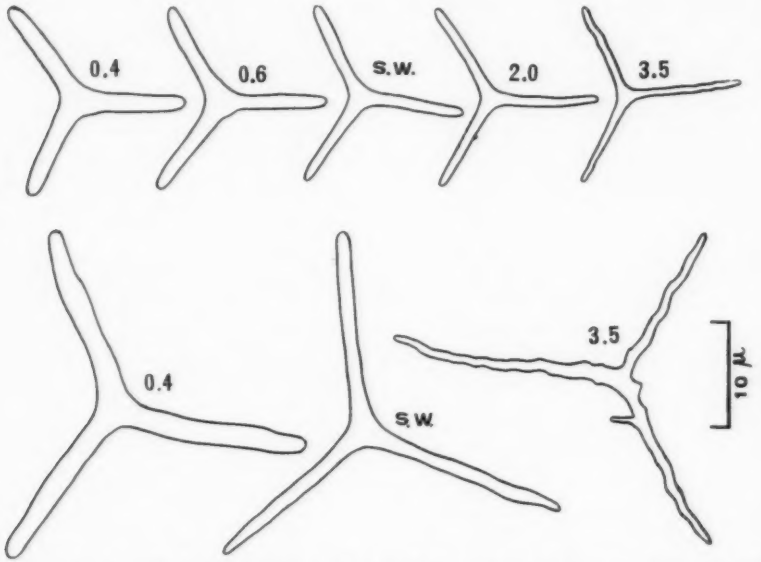

FIGURE 3. Camera lucida drawings of spicules in media of various Ca concentrations at two stages of skeletal development. Note correlation between Ca decrease and thickening of spicules. Larvae transferred to experimental media from sea water at gastrula stage with rudimental spicule granules.

to the dilution of Ca, while the increase in Ca content had only a slight effect in this sense. In considering the delay in skeleton formation at extreme concentrations, the retardation of the developmental rate must also be taken into account.

III. Form of the skeleton in calcium-low and -high media

When larvae were cultured in media with various Ca concentrations from a stage before the beginning of skeleton formation, the forms of the skeletons of these larvae at "the final stage" [3] showed characteristic features according to the calcium content, as is illustrated in Figures 1 and 2.

[3] By "the final stage" is meant the stage in which the larval skeleton reaches its maximum of development in foodless culture. Control larvae at this stage have four long arms, a func-

On the whole, in the range of 0.4–6.0 Ca, the skeletal forms of the experimental plutei were roughly similar to those of the controls (Fig. 1). If they are compared in detail, however, a slight shift can be recognized in the thickness and length of the skeleton, and in the shape and number of hooks, paralleling the concentration shifts in calcium. The thickening of the skeleton was inhibited in proportion to the increase in the Ca content of the medium; this was noticeable even as early as the tri-radiate spicule stage, as is indicated in Figure 3. The elongation of the skeleton

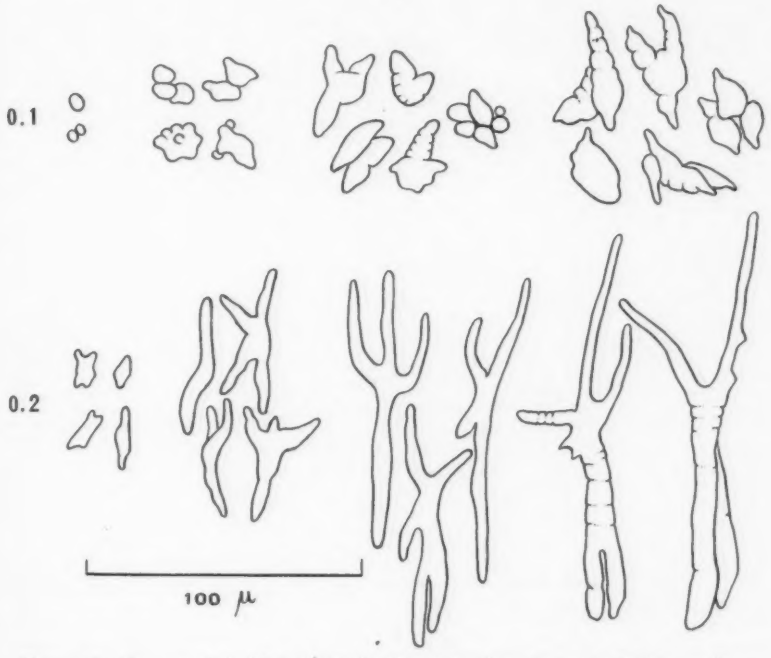

FIGURE 4. Process of skeletal growth in *H. pulcherrimus* larvae in Ca-low media (0.1, 0.2 × Ca).

was inhibited in proportion to the decrease in Ca concentration within the range 0.1–0.4 Ca (see Figs. 1, 2). There was a corresponding reduction in the number and the sharpness of the hooks, if the calcium content of the medium was reduced, and finally in 0.1 Ca, the hooks were missing.

At extreme concentrations outside the above range, there occur distinct differ-

tional digestive tract and a pair of coeloms. Since, however, the destined length of life of the experimentals turns out to be as long as that of the controls or even longer in some solutions, the data of the controls and experimentals are roughly comparable. When experimental larvae died before the end of the experiment, they were omitted from the data.

ences, with respect to the form and developmental process, between the skeletons of controls and of experimentals, as will be described below.

Skeletons in media extremely low in calcium. As was mentioned above, doubly refractive skeletons could be formed in larvae reared in such Ca-low media as 0.1 or 0.2 Ca from a stage before skeleton formation. In such media, these doubly refractive skeletal rudiments never developed into the tri-radiate form found in the controls. However, the rudiments continued to grow in size as irregular masses (Fig. 4). As a natural consequence, the skeletons at the final stage of such Ca-low larvae were extremely atypical.

On the other hand, if the larvae were transferred to 0.1 or 0.2 Ca after the formation of tri-radiate spicules, new skeletal development in the Ca-low media was

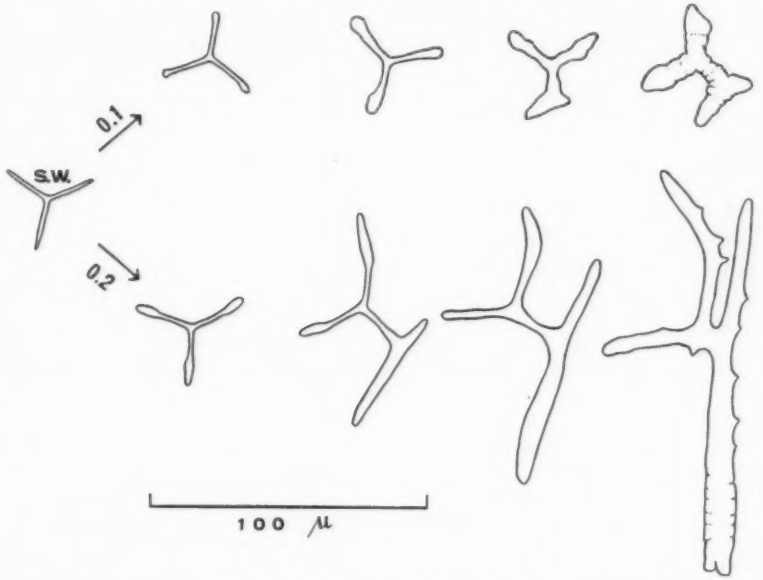

FIGURE 5. Modification of shape of tri-radiate spicules on transfer to Ca-low media (0.1 and 0.2 Ca).

recognized as thickenings at each of the three spicule tips, globular in 0.1 Ca and ovoid in 0.2 Ca. (This fact may indicate that in the normal process of skeleton formation, Ca deposition is more vigorous at the tips than at the other points of the skeleton.) Subsequently, all parts of the spicules gradually thickened until skeletons at the final stage resembled typical pluteus skeleton, although they were much dwarfed (Fig. 5).

If the larvae were transferred to Ca-low media after the pluteus stage, the thickening of the skeleton was found first at all tips and hooks of the skeletons (Fig. 6). In all cases in the two media lowest in Ca, it was a conspicuous feature of skeletal growth that the proportion of length to thickness was very small, par-

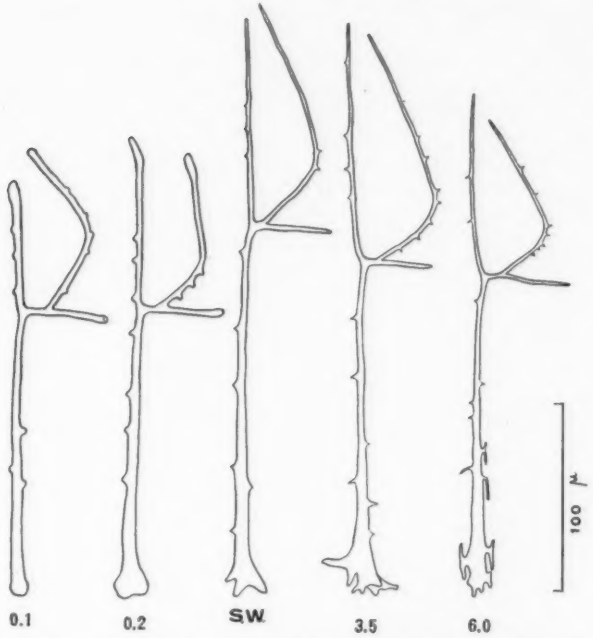

FIGURE 6. Effect of late transfer of *H. pulcherrimus* plutei to experimental media. Note effects of Ca change on form of hooks and skeletal tips.

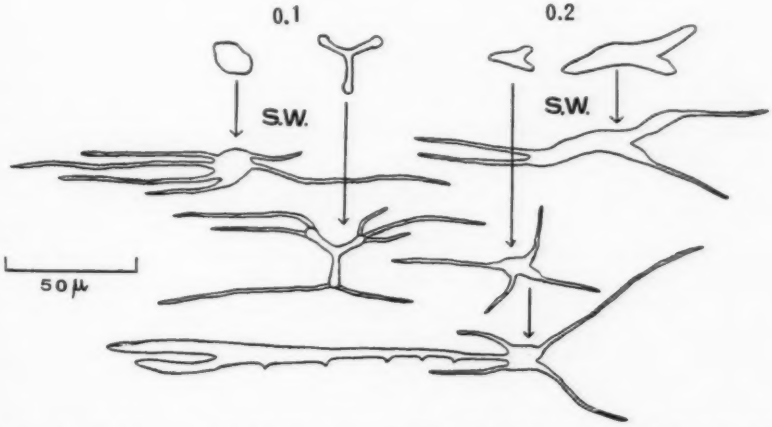

FIGURE 7. Skeletons of *H. pulcherrimus* larvae transferred back to sea water from Ca-low media (0.1 or 0.2 Ca), showing terminal elongation of slender skeletal rods.

ticularly in 0.1 Ca, and the surface of the skeleton became uneven as it thickened, as is shown in Figures 2, 4 and 5.

As a converse experiment to the above cases, if larvae which had been in 0.1 or 0.2 Ca were returned to sea water during the course of skeleton formation, several spindly processes invariably grew out from the edge of the massive skeleton, without regard to the stage of transfer (Fig. 7). In this case, if the larvae were transferred to 0.4, 0.8 or 1.0 Ca (sea water), the shape of the parts newly formed on the old one became longer and thinner in proportion to the new concentration of Ca.

Skeletons in extremely high calcium media. If the larvae were cultured in 10.0 Ca from a stage before skeleton formation, several spicule centers were formed, each of which developed into a tri-radiate spicule. These rarely attained a sufficient size to serve as functional pluteus skeletal parts (Fig. 2). Such abnormality was sometimes obtained in 6.0 Ca larvae. However, if sea water larvae were transferred to 6.0 or 10.0 Ca after the formation of tri-radiate spicules, excess centers of the spicules did not form and new skeletal development was continued to form the typical pluteus skeleton on the basis of the old spicules. In either case, the usual thickening failed to take place in such skeletons and there was also some reduction in elongation.

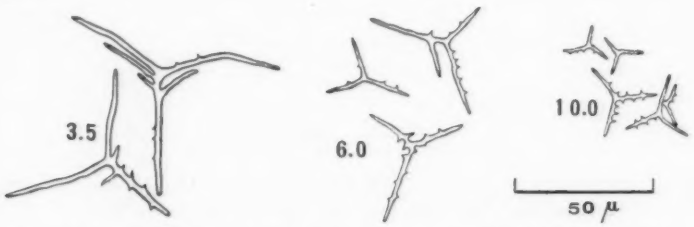

FIGURE 8. Spicules of *Hemicentrotus* gastrulae having precocious hooks in Ca-high media.

In 6.0 or 10.0 Ca, the side hooks of the skeleton which normally appear at the pluteus stage were formed prematurely at the gastrula stage, *i.e.*, at the tri-radiate spicule stage (Fig. 8). Such precocious development of the side hooks was also frequently observed in 3.5 Ca. If the transfer to Ca-high media was done after the pluteus stage, the shape of the hooks became sharper and longer as the Ca content of the medium was increased (Fig. 6).

IV. The effect of Ca concentration on apparent increase in length of the skeleton

Putting aside the aberrant skeletons formed in the two extremes of the Ca range, a comparison of the skeletal growth by microscopical measurement was made within the range of 0.4–3.5 Ca. Since the skeletal growth of individual larvae was very variable even among control larvae derived from the same batch, and further, since these individual differences became increasingly greater with the increasing divergence of the Ca concentration from that of sea water, particularly on the dilute side, the data given are averages of specimens showing good development (see Glaser, 1950).

To obtain uniform initial length of the spicules, the larvae were allowed to de-

velop in sea water until the formation of the tri-radiate spicules had taken place, before they were transferred into media of the above range of calcium, and the lengths of the spicules in the various media were compared at intervals thereafter. In spite of variation in the details of the apparent skeletal growth, the general trend showed that a high concentration of calcium caused better skeletal growth

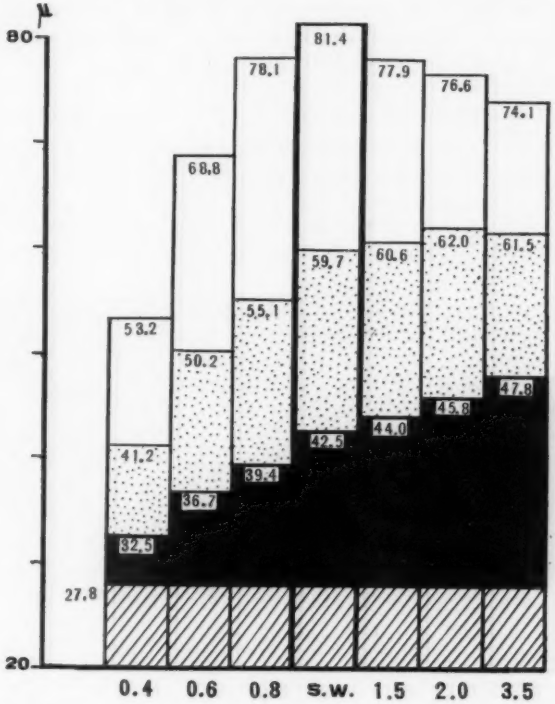

FIGURE 9. Apparent skeletal growth following transfer from sea water to media with various Ca concentrations. Material: *Hemicentrotus pulcherrimus* gastrulae. Ordinate: actual lengths of spicules calculated from camera lucida drawings. Abscissa: Ca concentrations of media. Columns with shading indicate initial length of spicules at transfer. Black, dotted and white columns indicate increase in skeletal length during each two hours after transfer at 16.5° C.

than sea water for a short time following transfer, but became harmful later on, while low calcium was relatively harmless.

The data of one experiment presented in Figure 9 illustrate this tendency. In this case, the length of the skeleton reached during the first two-hour period after transfer increased corresponding to the Ca concentration.[4] For the second

[4] Maximum increase in skeletal length during the first period was found sometimes in 1.5 Ca or in 2.0 Ca or in 3.5 Ca. In all cases, however, the skeletons formed in Ca-high media were longer than those in sea water.

two-hour period, however, the growth rate of the skeleton in the Ca-high media came down to approximately the same level as that of sea water, and for the third two-hour period, the increase in skeletal length in Ca-high media became inferior to that in sea water. On the other hand, the growth rate in Ca-low media decreased in proportion to the decrease in Ca concentration at first, but improved later.[5]

If the total lengths of post-oral rod + body rod at the final stage in various media were compared, the skeletons in 0.6 or 0.8 Ca were usually longer and in Ca-high media were shorter than the skeletons in sea water.

V. Spatial relations between ectoderm and skeleton

At the time when tri-radiate spicules were formed in sea water, spicules had not yet been formed in 0.1 or 0.2 Ca, but the ectoderm at the position of the presumptive post-oral arms was wrinkled (Fig. 10). If such larvae were transferred to sea water, the wrinkled ectoderm gradually expanded and formed the arm, accompanied by development of the spicules. However, if the larvae were kept in 0.1 Ca, the

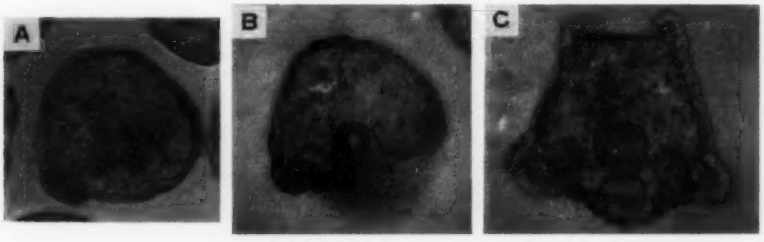

FIGURE 10. *H. pulcherrimus* larvae cultured in Ca-low medium. A, B: 0.1 Ca gastrulae, showing wrinkles of the ectoderm at position of presumptive post-oral arms. A: side view. B: vegetal view. C: 0.2 Ca pluteus, showing post-oral and antero-lateral lobes without rods.

wrinkles became deeper as development advanced, and frequently the wrinkled ectoderm fell off from the body as small vesicles, although in 0.2 Ca, the spicules eventually developed and short arms grew out.

At the stage in which the antero-lateral arms developed in sea water larvae, the ectoderm covering the area of the presumptive antero-lateral arms of 0.1 or 0.2 Ca larvae formed a process which could virtually be considered as an antero-lateral lobe, in spite of the fact that the antero-lateral rod was experimentally suppressed in these larvae (Fig. 10). On transfer back to sea water, this lobe developed into an antero-lateral arm supported by an attenuated skeleton. If several of these slender skeletal extensions grew simultaneously from the central mass, a single branch which elongated in the direction of the lobe acted as a substitute for the antero-lateral rod, but arms never grew out where other branches reached the ectoderm. On continuous culture in 0.1 or 0.2 Ca, the antero-lateral lobe gradually disappeared instead of being cut off from the body. Rulon (1941) reported that *Dendraster excentricus* embryos treated with NaSCN developed into larvae having the general

[5] The growth of the skeleton during the third period was frequently better in 0.8 Ca than in sea water.

shape of plutei in spite of the lack of the mesenchyme cells and a gut. The Ca-low larva reported here (Fig. 10) is very similar in shape to this larva of Rulon's (Fig 12 in his paper).

From the above fact, it can be said that the ectoderm which is to develop into the arm is able to self-differentiate, but for a normal arm to result, a spatial relation between the ectoderm and the skeleton is necessary. This has already been pointed out by Jenkinson (1911), MacBride (1914), Runnström (1931), v. Ubisch (1933) and Hörstadius (1935).

Discussion

As was described in Section II, the higher the concentration of calcium ions, the earlier is the time at which skeleton formation begins. From this fact it is to be expected that the skeletal growth in Ca-high media will be faster than in normal or Ca-low sea water. Such a situation, however, was found to obtain only for a short while after the calcium change (first two hours in Fig. 9). After this period, the skeletal growth was rather accelerated in such low concentrations of calcium as 0.6 or 0.8 Ca, and inhibited by high concentrations. It is still impossible to consider the above facts in terms of the rate of Ca deposition, since it is doubtful whether the apparent size variation of the skeleton is proportional to the quantity of deposited calcium. Abe and co-workers (1954) have reported on calcium deposition by adult oysters which were kept in media of different concentrations of radio-active calcium; if twice as much calcium was provided calcium deposition was slightly increased, while in the medium of half calcium, the deposition of calcium was reduced to nearly one-tenth that found in sea water.

There is another situation to be considered in sea urchin larvae. The spicules of these larvae always grow along a row of primary mesenchyme cells. This fact suggests that the direction of spicule elongation is decided by the mode of arrangement of the mesenchyme cells, and the length of the skeleton is conditioned by the distance to which they extend. The primary mesenchyme cells are further apart in low calcium because of the larger blastocoelic volume, and are brought closer together in high calcium by the smaller size of the blastocoel. On such a basis, it becomes understandable why the apparent growth of the skeleton in 0.6 or 0.8 Ca was rather superior, and that in Ca-high media was inferior to the growth of the controls.

Woodland (1906) and Prenant (1926) suggested from observations of normal skeletal development that a sheath-like structure which is found along the row of primary mesenchyme cells may be a matrix for the skeleton. If this is true, it must be concluded that the apparent length of the skeleton is conditioned by the length of the skeletal matrix which, in turn, is proportional to the distance covered by the longitudinally arranged mesenchyme cells. And further, the tendency that the ratio of length to thickness of the skeleton becomes smaller as the amount of available calcium decreases will find its explanation in the deformation of the matrix resulting from the change in the calcium content of the medium. A detailed description of the skeletal matrix will be reported in the next paper.

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Summary

1. The threshold Ca concentration for the growth of the larval skeleton is lower than that which permits the normal development of sea urchin larvae.

2. The time of the beginning of skeleton formation is retarded by a decrease in Ca concentration from the normal level, while an increased Ca concentration has little effect in this respect.

3. Modification of the Ca concentration of the medium influences the form of the skeleton of sea urchin larvae.

a). In larvae transferred to very dilute calcium before the time of skeleton formation, a mass of unorganized skeletal substance develops.

b). In larvae put into very concentrated calcium from a similarly early stage, several tri-radiate spicules are formed in each larva, but these fail to make a typical skeleton.

c). If the transfer is deferred till the tri-radiate spicules are formed, new skeletal development in the experimental media is continued on the old spicules, and a pluteus skeleton which is similar to the typical one results, though it is much stunted in Ca-low, and slender in Ca-high media.

d). The proportion of the thickness to the length of the skeleton is inversely proportional to the Ca concentration. This is true regardless of the stage of transfer.

e). The elongation of the skeletal rods is inhibited at the extremes of the Ca-series, particularly on the dilute side.

f). The apparent increase in skeletal length is rather better in 0.6 or 0.8 Ca than in high-calcium, except that the contrary situation holds for a short time after transfer.

4. The ectoderm of the presumptive arm may differentiate without any skeleton. However, such differentiated ectoderm finally falls off from the body or disappears unless the supporting skeleton develops.

5. It is suggested that an organic matrix may be formed before deposition of the mineral components of the skeleton; that this matrix is deformed by changes in the calcium concentration; and that such deformation, in turn, influences the rate of growth and the form of the skeleton.

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CYTOKINESIS OF MULTI-SPINDLE CELLS

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Although in cell division it is usually apparent that the position of the constriction furrow is related to that of the spindle, the relationship of the two is not clear. Harvey (1935), working with centrifuged sea urchin eggs, came to the conclusion (p. 296) that, "Since a cleavage plane can come in without relationship to the mitotic figure, it would seem that the mitotic figure itself is an expression of more fundamental physical and chemical changes taking place in the cytoplasm and surface of the egg, and not a cause in itself of the cleavage process." Waddington (1952), working with frog eggs, stated (p. 488) that, "The only evidence, in the present experiment, suggesting an influence by the spindle on the cleavage furrow is that of the few eggs described in which an attempt to move the spindle was followed by an alteration in the course of the furrow. General considerations derived from a study of the cleavage of eggs of many groups certainly suggest that the location of the spindle ultimately determines the position in which the furrow appears." Carlson (1952), on the basis of microdissection studies on grasshopper neuroblasts, held (p. 217) that, "Formation of the cleavage furrow is independent of the spindle and chromosomes, but its position depends to a limited extent on their position at late anaphase." The writer (Roberts, 1955), working with the same material, has presented evidence suggesting the spindle poles, through their relationship to expansions of the polar cell membranes, play an important part in determining the site of the constriction furrow. Cornman and Cornman (1951) have suggested that, upon the dissolution of the nuclear membrane, nuclear material gathers around the centrosomes, is transported by the asters to the cell surface, and is carried by currents to the equator, where it induces the establishment of the furrow. Marsland (1939) and others have described the existence of a "plasma-gel girdle" around the equator of the sea urchin egg prior to cytokinesis without relating it in any way to the spindle.

Cells containing several spindles, usually two to four, are regularly found in preparations of isolated living testicular cells of *Popilius disjunctus*. As is usual in this material, the spindles are invested by long filamentous mitochondria from pole to pole, making the spindles strikingly visible, especially with phase microscopy. Such a multi-spindle condition has been observed in spermatogonia and primary and secondary spermatocytes, which are considerably larger than their single spindle counterparts. Conditions in these cells differ from those seen in dispermic and parthenogenetic marine eggs in that they arise from regularly occurring multinucleate cells, and, ordinarily, each spindle has its own centrosomes and chromosome complement. During cytokinesis each cell divides into two or more cells, de-

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pending on the number of spindles and the orientation of the spindles with respect to each other and to the cell membrane. The patterns of cytokinesis which are observed with varying spindle orientations provide a means for studying the role of the spindle poles in the establishment of constriction furrows.

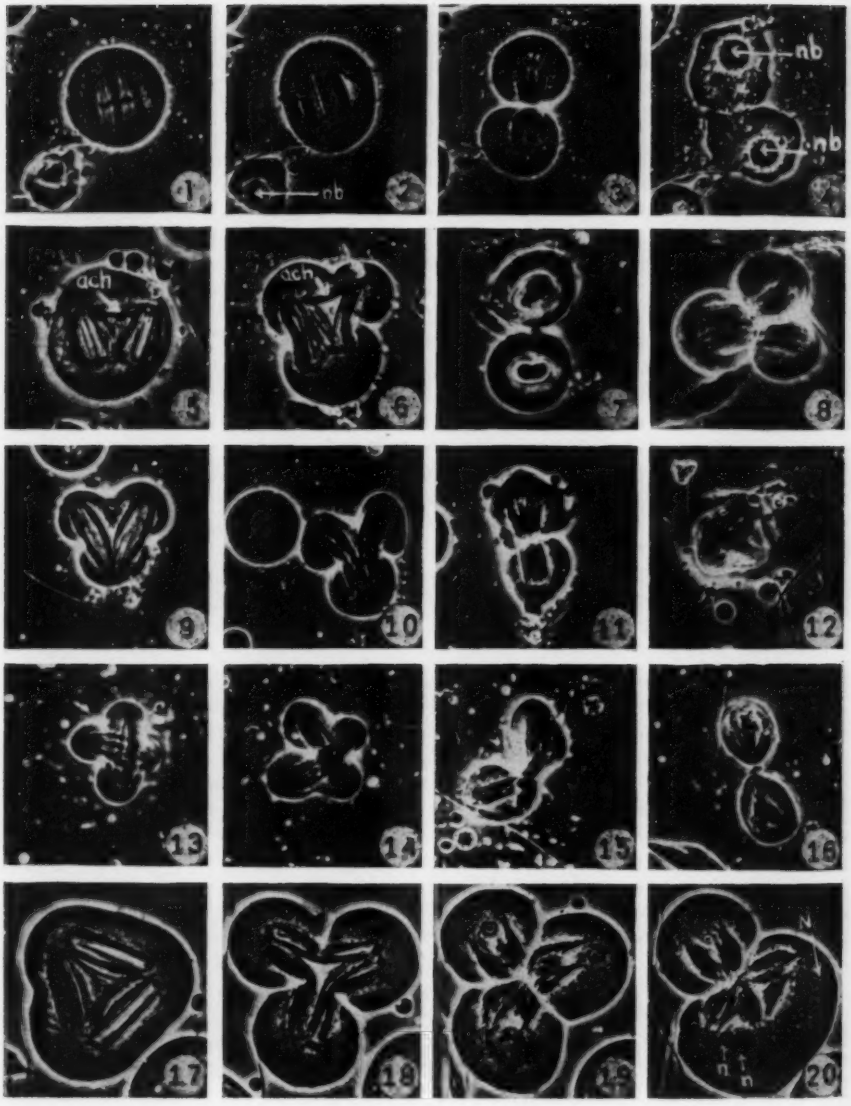
Materials and Methods

Beetles were collected from rotting hardwood logs, brought into the laboratory, and maintained by supplying moist pieces of wood from the logs where they were collected. During the colder months best results were obtained from beetles which had been kept at room temperature, 22° C., for at least one week. Prolonged exposure to temperatures above 30° C. resulted in an absence of dividing cells in the testes. With proper temperature and humidity conditions, dividing cells may be obtained during the entire year.

Several media have been used for the culture of isolated beetle cells. Whole hemolymph is unsatisfactory because it rapidly darkens and becomes cytolytic upon exposure to air. The addition of purified phenyl thiourea prevents blackening and reduces the cytolytic effect, but cell division is adversely affected. A better medium is obtained by flash heating hemolymph, which inactivates those factors responsible for blackening and cytolysis, but also coagulates and precipitates proteins. Cells suspended in such deproteinated hemolymph divide fairly well, but the preparations do not last as long as is desirable. The modified insect Tyrode solution used by Gaulden and Carlson (1951) is satisfactory for preparations which are to be studied for short periods of time, 4 to 6 hours. Best results have been obtained by using a mixture of the insect Tyrode solution with deproteinated hemolymph. The proportions are not critical; usually 50% to 75% deproteinated hemolymph has been used. Preparations made with this medium usually last 18 to 24 hours, and permit observation of two complete cycles of division.

The preparation of this medium presents several difficulties and therefore will be described in some detail. A sterile one-ml. tuberculin syringe equipped with a 1½ inch 26-gauge needle and containing 0.05 ml. of insect Tyrode solution is held in readiness. A beetle, preferably male, is selected and the anterior portion of the head, at the level of the dorsal horn, cut off by one swift cut with heavy scissors. Hemolymph from the severed dorsal vessel accumulates in large clear drops. If the body of the beetle is not squeezed, there is usually no contamination of the hemolymph by material from the digestive tract. If contamination does occur, it can be detected by a brown color in the otherwise colorless fluid, in which case it must be discarded. Occasionally, compression of tissue blocks the dorsal vessel, but this can usually be overcome by slight probing with the syringe needle. The drops of hemolymph are drawn into the syringe and mixed with the Tyrode solution. Ordinarily, enough hemolymph can be obtained to make the total fluid volume in the syringe up to 0.15 ml. It is essential to work rapidly, since the blackening occurs within a few minutes and renders the fluid useless. The fluid in the syringe is discharged into a thin-walled glass bubble for flash heating. The bubbles used are blown from Pyrex tubing and are about 15 mm. in diameter with a capillary neck about 20 mm. in length. The diameter of the neck is just sufficient to admit the 26-gauge needle. After introduction of the fluid, the tip of the neck is sealed

PLATE I



FIGURES 1–20.

in a flame and the bubble is plunged into hot water, just below the boiling point, for 45 seconds. It is convenient to have a beaker of boiling water available and to remove it from the heat while the bubble is being sealed. The fluid within the bubble must be agitated as it enters the hot water and during the entire heating process, otherwise, it will gel and no free fluid can be obtained. In successful preparations the precipitated proteins adhere to the wall of the bubble. After cooling, the bubble should be rotated so that any droplets of distillate are incorporated into the fluid.

Two pairs of testes are present in the adult male beetle. Ordinarily, one pair will supply sufficient material for the amount of medium available. The testes are dissected out and placed in a small dish of Tyrode solution and adherent tissue removed. It is important that the seminal vesicle be removed, otherwise the preparation will be filled with spermatozoa. Nematodes, which are abundant during the summer months, should be removed. The Tyrode solution also has the important function of washing the testes free of unaltered hemolymph which, if carried into the final preparation, has a deleterious effect. The testes are transferred from the Tyrode solution to the center of a sterile microscope slide, carrying over as little fluid as possible. They are then covered with about half the prepared medium, which has been withdrawn from the bubble with a sterile one-ml. tuberculin syringe and a 26-gauge needle. The testes are squashed against the slide, using flat-tip cover-slip forceps. Thorough squashing is necessary to rupture the cysts of the testis, but a scouring motion, which destroys many cells by its shearing action, should be avoided. The remainder of the medium is discharged over the squashed testes and the slide tilted so that the fluid and cells drain away from the squashed tissue. This cell suspension is then drawn into the same syringe, leaving the fibrous debris on the slide.

One-centimeter squares are outlined on coverslips with vaseline extruded from a 26-gauge needle. Each square is flooded with the prepared cellular suspension

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FIGURES 1–4. Division of a secondary spermatocyte containing two parallel spindles. A normal spermatid containing a single nebenkern (nb) is shown at the lower left of Figure 2. The two large spermatids resulting from the division are shown in Figure 4.

FIGURES 5–7. Division of a secondary spermatocyte containing two divergent spindles. A well developed achromosomal spindle (ach) connected the divergent poles. A second furrow formed between the divergent poles but soon regressed. Two large spermatids resulting from the division are shown in Figure 7.

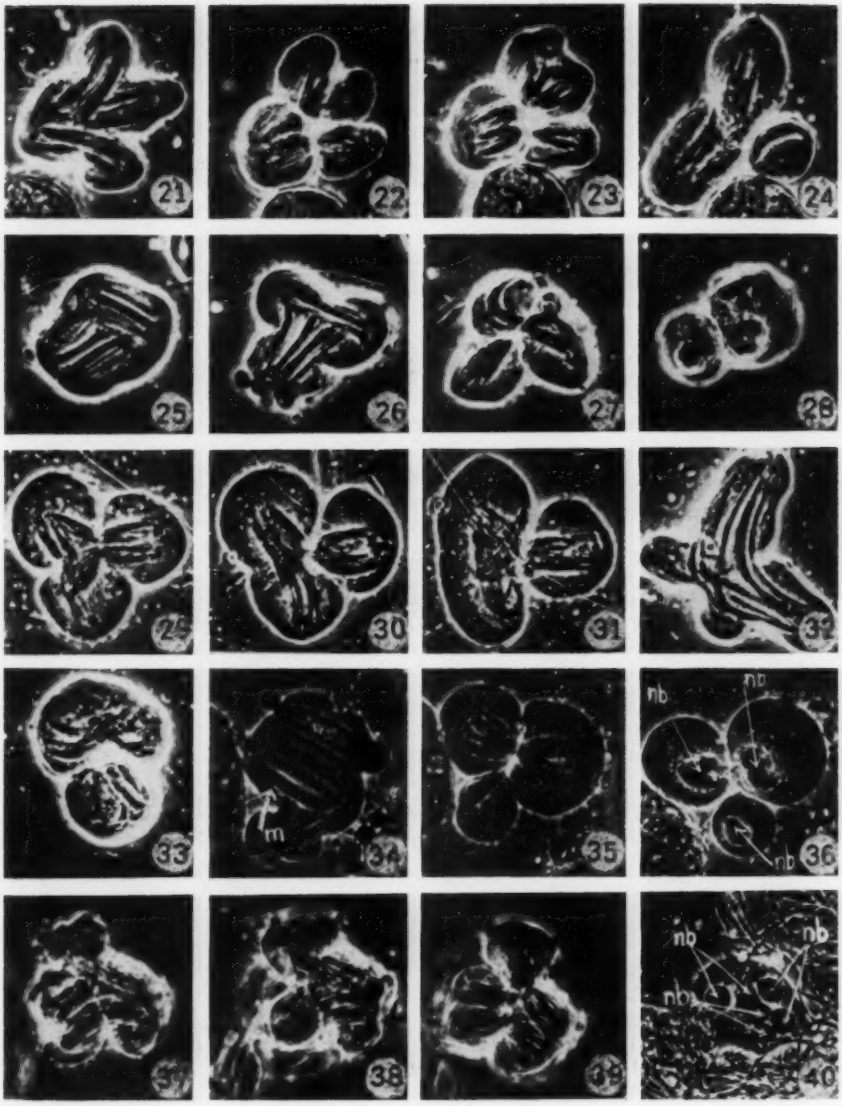
FIGURE 8. Three daughter cells resulting from division of a primary spermatocyte containing two divergent spindles.

FIGURES 9–11. Division of a primary spermatocyte containing two divergent spindles. There was no evidence of cross-connections between the divergent poles. A furrow developed between the divergent poles and advanced halfway before regressing.

FIGURES 12–16. Division of a primary spermatocyte containing two spindles at right angles to each other, in different planes and not intersecting. There was no evidence of cross-connections between the poles. Furrows developed between the poles and the cell appeared to be dividing into four. As the furrows impinged on the spindles their orientation changed so that they came to be approximately parallel and the cell divided into two.

FIGURES 17–20. Cytokinesis of a primary spermatocyte (?) containing three spindles. Furrows developed between the poles, in the equatorial region. In late cytokinesis one furrow regressed, and two of the daughter cells fused. In Figure 20 it can be seen that in one case the two chromosome groups fused, forming a large nucleus (N) and in another the groups remained separate, forming two small nuclei (n).

PLATE II



20 μ

FIGURES 21–40.

and the excess fluid withdrawn into the syringe. This gives a relatively flat film whose thickness can be adjusted as required. The coverslips are inverted over depression slides and sealed with vaseline or heavy mineral oil. Two types of depression slides have proved especially useful. One (Fisher-Littman) is of the same thickness as a standard microscope slide and has a shallow circular depression with a flat polished bottom. This slide permits the use of oil immersion phase objectives. A specially made plastic well slide has been most commonly used. This slide is too thick to permit the use of oil immersion objectives, but has the advantage that it takes 22 × 50 mm. cover-slips on which three vaseline squares can be outlined. The relatively large air space is compensated for by recesses on all four sides into which cotton, wet with Tyrode solution, can be packed.

Divisions were studied with dark contrast phase optics (Spencer) using long focal length phase plates, to permit study of thick preparations. In the writer's experience with living cells, the long focal length phase plates have proved to be clearly superior, regardless of the thickness of the preparation. Light intensity was controlled by a variable transformer and overheating of the slides was avoided by using a copper sulfate water cell and three heat-absorbing filters (Corning). Observations and records were made at room temperature, about 22° C.

Observations

The observations reported are based on spindle orientation in living cells as revealed by oriented filamentous mitochondria. The spindle fibers are not visible. A comparison with stained sections indicates that the structure observed in living cells is a fairly accurate representation of the actual distribution of fibers. The chromosome-bearing spindles are faithfully visualized, but cross-connections (achromosomal spindles) between the poles of adjacent chromosome-bearing spindles are less reliably indicated. The filamentous mitochondria, which are preserved and stained by those methods used to demonstrate fibers, tend to obscure the fibers along which they are oriented. Wherever oriented mitochondria indicate the existence of such cross-connections, fiber connections actually exist, and the relative abundance of fibers is correlated with the abundance of oriented mitochondria. In the absence of oriented mitochondria, a few weak fibers may be present or there may be none.

Figures 21–24. Cytokinesis of primary spermatocyte containing three divergent spindles. There was no evidence of cross-connections between the divergent poles. Furrows developed between the divergent poles, and the cell appeared to be dividing into four. Strong blebbing, Figures 22, 23, was followed by regression of one furrow, and the cell eventually divided into three.

Figures 25–28. Cytokinesis of a secondary spermatocyte containing three spindles. After cytokinesis was virtually complete, Figure 27, two daughter cells fused, giving rise to one large and one small spermatid.

Figures 29–33. Cytokinesis of primary spermatocytes(?) with three spindles.

Figures 34–36. Cytokinesis of a secondary spermatocyte containing four spindles, three parallel and the fourth divergent. Although a mitochondrion (m) was present in the area between the divergent poles, no achromosomal spindle was visible. Two large and one small spermatid resulted. Note the relative size of the nebenkerne (nb).

Figures 37–39. Cytokinesis of a cell containing six spindles. Three daughter cells resulted.

Figure 40. Spermatid containing six nebenkerne (nb). This cell was observed immediately after the preparation was made, far too short a period for this to be the result of the procedure used.

Figures 1–16 illustrate cytokinesis of cells containing two chromosome-bearing spindles. In the simplest situation the two spindles, each with its own chromosome complement, were parallel and close together. The furrow formed in the equatorial position and the cell constricted into two daughter cells as though only one spindle were present (Figs. 1–4). In some cases the two chromosome groups in each polar region fused to form one nucleus. In others, two nuclei were formed. In many double spindle cells the spindles were in the same plane but lay at an angle to each other. Under these conditions a furrow developed in the equatorial region, and a second furrow invariably developed between the divergent poles. The formation of the second furrow did not depend upon the presence of cross-connections between the divergent poles, as far as could be judged from the presence of oriented mitochondria. In some cases a well developed achromosomal spindle was present (Figs. 5–6), and in others (Figs. 9–10) there was little or no evidence of cross-connections. The fate of the second furrow depended primarily upon the distance between the divergent poles and not on the presence of fiber connections between these poles. Where the poles were only slightly separated, the second furrow soon regressed and the cell constricted into two daughter cells (Figs. 4, 7). Regression often occurred after cytokinesis was virtually complete, resulting in the fusion of two or even all three of the daughter cells. A few double-spindle cells have been observed in which the two spindles lay at right angles to each other, in different planes, and not intersecting (Figs. 12–16). During cytokinesis furrows developed between each of the four poles so that the cell appeared to be dividing into four daughter cells. In the cell illustrated there was no evidence of cross-connections between the poles. In cells of this type, as the furrows impinged upon the spindles their orientation changed so that they came to be approximately parallel, and the cell eventually divided into two.

Figures 17–33 illustrate cytokinesis of cells containing three chromosome-bearing spindles. Various spindle orientations occurred. In some, the three spindles lay in the same plane and formed the sides of an equilateral triangle (Fig. 17). Furrows developed in the equatorial region of each spindle and developed so that division into three daughter cells was virtually completed (Fig. 19). In late telophase, after re-formation of interphase nuclei, two of the daughter cells fused into one large cell (Fig. 20). In another case, first observed in early cytokinesis, the three spindles were arranged as shown in Figure 21. There was no evidence of cross-connections between adjacent poles. Furrows developed between each of the diverging poles and across the equatorial region of all three spindles. The cell appeared to be dividing into one large and three smaller daughter cells. Later, strong blebs developed on two adjacent incipient daughter cells (Figs. 22, 23), the furrow between them receded, and the cell finally divided into two large and one small daughter cell (Fig. 24). It is noteworthy that the appearance of strong blebs adjacent to a furrow always resulted in the regression of that furrow. In another case two of the three spindles were parallel and the third at approximately right angles to the other two (Fig. 25). Furrows formed in the equatorial regions and advanced until, in late cytokinesis, two incipient daughter cells fused so that one large and one small daughter cell were produced (Fig. 28). Other spindle orientations in which the spindles were, in part, in different planes are illustrated in Figures 29–31 and 32–33. As in the other cases described, furrows developed between

the divergent poles without regard to the presence or absence of cross-connecting fibers.

Cells containing from four to six chromosome-bearing spindles have been observed. Cytokinesis in such cells is complex and difficult to follow (Figs. 37–39). A simpler case of a cell with four chromosome-bearing spindles is illustrated in Figures 34–36. It is apparent here also that furrows develop between spindle poles and not necessarily in relationship to the equatorial region of the cell or spindle.

Discussion

It is evident in this material that the site of furrow formation is related to, and apparently dependent upon, the position of the spindles. It has been repeatedly observed that furrows form approximately halfway between spindle poles and not necessarily in relation to the equators of the spindles. The presence of spindle fiber tracts (chromosomal or achromosomal spindles) between the poles does not appear to be essential for the formation and further development of a furrow. Since in the usual situation the equator of a spindle does lie halfway between spindle poles, the commonly observed relationship between furrow and spindle equator can be explained on this basis.

Bajer (1954) has described a quite similar situation in primary spermatocytes of the snail *Cepea hortensis*. Although his figures of trinuclear and tetranuclear cells show only the chromosome groups and not the spindles, it is clear here also that furrows form between spindle poles and not in relationship to the equators. His description of cytokinesis of binuclear cells without division of the nucleus is especially striking. In these cells each nucleus reached metaphase, with the two spindles parallel and widely separated. After a "long" metaphase with "quiverings" of the plate, the furrow formed and advanced between the two spindles without cutting through either. The cell divided into two and interphase nuclei were reconstituted from each metaphase configuration.

These observations support the idea that the spindle poles initiate furrow formation, possibly through a "furrow determiner" as postulated by Cornman and Cornman (1951), or a "structural agent" as proposed by Swann (1952) and Mitchison (1952) and modified by Roberts (1955). They do not appear to provide an adequate basis for deciding between these alternatives. Bajer's observations on cytokinesis of binuclear cells without nuclear division would appear to support the view of the Cornmans that nuclear material released upon the dissolution of the nuclear membrane may act as a furrow determiner. It has been noted that in multi-spindle cells the distance apart of divergent poles influences the furrow which develops between them. When the distance is sufficiently great, the furrow advances and may succeed in dividing the cell. This does not appear to support the view of the Cornmans, since it would seem that the accumulation of large quantities of "furrow determiner" in the small area between the poles should result in the development of a strong furrow. Swann and Mitchison postulate that the furrow is established by an initial contraction of the cell membrane between the "structural agent" induced polar membrane expansions, and that spread of the expansions into the furrow actively advances it. In this material it has been observed that when large blebs, which are membrane expansions of a sort, occur in the furrow area,

the furrows usually regress. Further, it would seem that proximity of a developing furrow to the point of release of the expansion inducing substance should facilitate the further growth of the furrow. There is no difficulty if the furrow, after a critical stage in its establishment, is autonomous and continues to develop by contraction or autocatalytic growth as suggested by the Cornmans. This is supported by the work of Waddington (1952), Harvey (1935), Carlson (1952) and others. The observation that the furrow developing between spindle poles separated by only a short distance soon regresses could be explained as the result of the initial contraction being obliterated by the approaching waves of expansion before the critical stage is reached.

In the present work, as in that of the Cornmans, there is evidence of a critical stage just at the completion of cytokinesis. Where multiple furrows are present, it is usual that one or more furrows regress, and it is rarely that these cells succeed in dividing into more than two daughter cells. This was also true of the multinuclear cells described by Bajer. Certainly adequate spindle material is available, and it is not likely that this is a factor as suggested by the Cornmans. Bajer's observations indicate that spindle material is not required for the completion of cytokinesis, since constriction was completed without the furrow cutting through a spindle.

It is evident that some unusual chromosomal relationships must result from divisions such as those described. These have not thus far been analyzed. Preliminary attempts to do chromosome counts by the squash method have not been satisfactory. The chromosomes are so small that the pressure required to spread them sufficiently for counting made cell boundaries unrecognizable, and it was not possible to determine whether adjacent chromosome sets came from the same or different cells.

The multi-spindle cells studied do not result from the procedures used. Prometaphase lasts approximately three hours, and anaphases and telophases have been observed within a few minutes after making a preparation. Living spermatids have frequently been observed to receive as many as two sets of chromosomes (Figs. 4, 7, 28) and occasionally as many as three (Fig. 36). In the normal spermatid the single nebenkern divides into two before the mulberry stage is reached, so that not more than two nebenkerne are normally seen. Spermatids containing at least six nebenkerne (Fig. 40) have been observed in freshly made preparations.

Summary

1. Patterns of cytokinesis have been studied in living multi-spindle cells obtained from beetle testes.

2. Furrows develop between spindle poles, whether or not connected by spindle fibers, and not necessarily in relationship to the equator of a spindle.

3. The further development of furrows is related to the distance apart of spindle poles. If the spindle poles are close together, the furrow soon regresses; if the poles are sufficiently far apart, the furrow advances and may succeed in dividing the cell.

4. When large blebs develop in the furrow area, the furrow regresses.

5. The observations reported are discussed in the light of current theories of cytokinesis.

6. Details of the unusual chromosome distributions which result have not been analyzed in detail.

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RE-EXAMINATION OF THE PRODUCTION OF CYCLOPIA IN FUNDULUS HETEROCLITUS WITH MAGNESIUM CHLORIDE AND ETHYL ALCOHOL [1]

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During three seasons (1948, 1949, 1955) attempts were made to produce perfect cyclopean fish for anatomical and physiological studies. Because of commonly accepted, misleading statements in the literature, much time was wasted at first. In brief, the large percentages of cyclopean embryos reported after $MgCl_2$ treatment by Stockard (1907, 1909) are not readily obtainable by this method; but instead, very large numbers of experimental animals must be handled to secure even a few hatched, perfect cyclopean fish.

The first purpose of this paper is to present quantitative data on this negative aspect of the problem. Such data have been lacking in previous reports, although McClendon (1912) has pointed out that the high percentages obtained by Stockard were not obtained by him. A second, positive purpose is to suggest that specific temperature in combination with the chemical treatment is of prime importance. Third, since ethyl alcohol treatment gives a large percentage of cyclopean embryos (although they are extremely unlikely to hatch), precise data on the effective concentrations are presented.

Magnesium Chloride

In work with this salt, gram molecular stock solutions of $MgCl_2 \cdot 6\ H_2O$ were made at the start of each experiment. In the manner of Stockard, 18 parts of this solution were added to 42 parts of sea water, 19 parts to 41 parts of sea water, etc. These were designated by Stockard as 18/60 *M*, 19/60 *M*, etc. The fact that they do not truly bear this relation to a molar solution is not especially significant. If a series of concentrations from completely ineffective to completely lethal is used, the optimum concentration will be included. The method is convenient and allows close comparison with Stockard's work.

In 1948 the experiments used fish obtained May 21st from a live-bait dealer in Boston, and sea water was obtained off the coast away from the city and stored in glass carboys. A liter of stock solution was made with 203.33 grams of $MgCl_2 \cdot 6\ H_2O$ in distilled water. About half an inch of properly diluted solution was added to each batch of eggs in 4″ finger bowls from 3 to 18 minutes after insemination. The bowls were stacked to prevent undue evaporation and stored in

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TABLE I

1948 $MgCl_2$ experiments

Solution	Dead or undeveloped	Two-eyed embryos	Cyclopean embryos	Double embryos
18/60 $MgCl_2$ (2 bowls)	675	7	0	0
19/60 $MgCl_2$ (8 bowls)	1081	261	2	1
20/60 $MgCl_2$ (2 bowls)	338	31	0	0
Control (sea water)	62	0	0	0

an aquarium room with a temperature range of 16 to 21° C. Solutions were replaced every 48 hours. Undeveloped but dead eggs were counted and removed after the first two days. After 12 days the embryos were preserved and inspected under the compound microscope. The results are presented in Table I.

In 1949 eggs were fertilized at Woods Hole from June 16th to July 5th. The season was early and it became increasingly difficult to find fish which had not already shed. At first fish came from the waters around Woods Hole, but during the last week some were obtained from Barnstable on the north side of Cape Cod.

Sea water from the outlets on the water tables in the laboratory was added to a depth of half an inch, five to ten minutes after fertilization, and replaced by magnesium chloride solutions when the embryos were in the four- to eight-cell stage. Bowls were tipped up and drained as dry as possible in changing solutions, to avoid

TABLE II

1949 $MgCl_2$ experiments

Solution	Died during development	Normal two-eyed embryos	Cyclopean embryos
A. Stock solution made up in sea water			
15/60 $MgCl_2$	3 (12%)	23	0
18/60 $MgCl_2$	21 (36%)	37	0
22/60 $MgCl_2$	35 (81%)	8	0
B. Stock solution made up in distilled water			
15/60 $MgCl_2$	7 (18%)	33	0
16/60 $MgCl_2$	8 (18%)	37	0
18/60 $MgCl_2$	4 (7%)	51	0
19/60 $MgCl_2$	30 (26%)	84	0
20/60 $MgCl_2$	42 (41%)	60	0
21/60 $MgCl_2$	70 (77%)	21	0
23/60 $MgCl_2$	76 (77%)	23	0
25/60 $MgCl_2$	51 (100%)	0	0
C. Sea water controls (adults for second control came from closed brackish pond)			
	2 (6%)	30	0
	52 (48%)	56	0

further dilution. The solutions were replaced every 12 hours; embryos were kept in the solutions for 48 to 72 hours, until the condition of their eyes was clear from inspection under the compound microscope. In contrast to the previous experiments, unfertilized eggs were removed within several hours of insemination; therefore only eggs which passed through the initial cleavage stages were tabulated. Finger bowls were stacked on a desk, and water temperature in the bowls was recorded at times scattered through the day and night. Of 50 recorded temperatures, 28 fell between 23° and 25° C. Only two were below 22° C. (20.5° and 21.0°) and four above 26° C. (26.25°, 26.5°, 27.25°, and 27.5°).

The effect of magnesium chloride dissolved in sea water alone (Stockard, 1907) was tested; 203.33 grams of $MgCl_2 \cdot 6\ H_2O$ were dissolved in one liter of sea water.

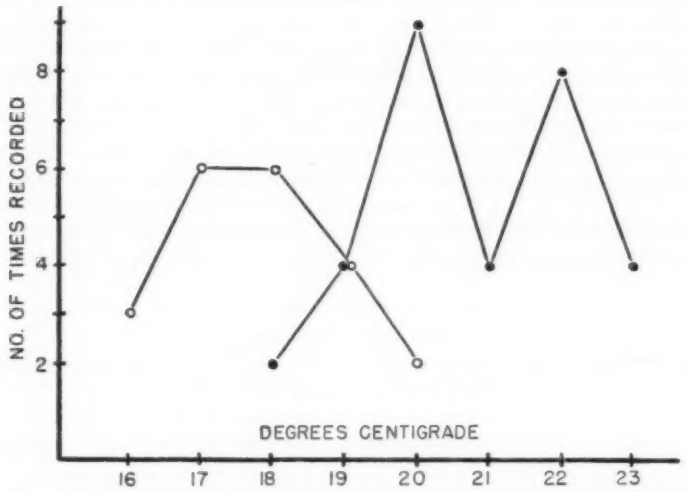

FIGURE 1. Recorded experimental dish temperatures, 1955. Temperature was recorded usually four times a day, and diurnal fluctuation in the laboratory room was kept as low as possible. Open circles represent recordings before June 12th, solid circles represent recordings after June 12th.

This is not a molar solution but the salts in sea water would cause inexactness anyway. Closer measurement seemed unnecessary since a range of concentrations was to be employed. This stock solution was then added to sea water in the same manner as the distilled water solutions of magnesium chloride had been. In another series, a gram molecular solution of $MgCl_2 \cdot 6\ H_2O$ in distilled water was titrated colorimetrically. This solution was added to sea water and the eggs and bowls were handled as described. The results of these two series are shown in Table II.

In 1949 because of the failure of the magnesium chloride treatment to produce any cyclopean embryos, more extensive series were not run. Alcohol treatment instead was used to secure cyclopeans for anatomical study (Rogers, 1952). The alcohol method had the disadvantage of not allowing embryos to develop well enough to hatch when used in concentrations that would produce cyclopia. Ac-

cordingly, when free-swimming cyclopean fish were desired for physiological tests in 1955, very large series of $MgCl_2$ experiments were carried out. From the results of 1948 and 1949 there was a suggestion that temperature might have an effect. The hurricane-flood damage of the previous season at Woods Hole prevented use of the cold rooms for some time in 1955, so experiments were carried out on a laboratory table at first. Room temperature was kept as constant as diurnal fluctuation allowed and experimental dish temperature was recorded at times scattered through the day and night. These experiments fall into two groups: those in which the embryos passed the critical eye-determining period during cold weather between June 5th and 11th (series A), and those after a storm temporarily

TABLE III

1955 $MgCl_2$ experiments

No. of bowls	Solution	Died during development	Normal two-eyed embryos	Cyclopean embryos	Other abnormals	% of living that were abnormal
			Series A			
3	15/60 $MgCl_2$	54—(31%)	120	0	0	
19	18/60 $MgCl_2$	682—(28%)	1713	38	22	3.4%
23	19/60 $MgCl_2$	864—(28%)	2139	58	60	5.2%
11	20/60 $MgCl_2$	1052—(51%)	967	17	38	5.4%
2	21/60 $MgCl_2$	136—(52%)	124	2(?)	1	
3	24/60 $MgCl_2$	514—(95%)	26	1(?)	1	
1	25/60 $MgCl_2$	153—(88%)	20	0	0	
2	Sea water controls	46—(10%)	395	0	0	
			Series B			
27	19/60 $MgCl_2$	Not recorded	3488	79	15	2.6%
			Series C			
	19/60 $MgCl_2$					
24	10° C.	1429—(100%)	0	0	0	
24	18° C.	292—(20%)	1070	42	26	6.0%
24	25° C.	137—(11%)	1156	3	7	0.9%

prevented obtaining adults, or the warmer period between June 12th and 19th (series B). The recorded temperatures during these two periods are plotted in Figure 1.

A third series was run in temperature-controlled chambers from June 22nd to July 3rd (series C). The 10° cold room varied from 6° to 10° C., but was between 9.5° and 10° C. most of the time. The 18° room varied between 16.5° and 19.1° C.; the 25° chamber, between 25° and 26° C. The two colder chambers were sizeable rooms, whereas the 25° chamber was the size of a household refrigerator. Twenty-four bowls were kept at each temperature. In 16 cases batches of eggs, each from single pair matings, were divided and a third of each batch placed in a

bowl at each temperature, to have a control on hereditary disposition as far as possible. The remaining eight bowls at each temperature had to be made up by dividing into thirds, batches of eggs combined from stripping many adults. After the eyes were determined, embryos were returned to sea water and kept at laboratory room temperatures (around 24° C. at that time).

Methods of handling all the bowls followed those described for 1949. A gram molecular solution of $MgCl_2 \cdot 6\ H_2O$ in distilled water was used in every case as the stock solution; no sea water stock solutions of $MgCl_2$ were employed. All of the 16,987 eggs were inspected under the dissecting microscope at least twice, once in the cleavage stages, when additional, non-developing eggs were removed, and again to classify the embryos after the eyes had developed. In addition, all 410 abnormal embryos were kept in sea water for a further period so that they could be more certainly classified after further development, although even then reasonable doubt sometimes remained. Complete records were kept on every bowl; the salient features of the 64 pages of data are summarized in Table III.

Alcohol

Stockard (1910) prepared mixtures on a percentage basis and found that from 3 to 9% ethyl alcohol in sea water produced viable cyclopean embryos, although he stated (p. 369) that "only a few of the alcoholic specimens ever develop sufficiently to hatch and swim about as do the Mg embryos." In his experiments batches of 60 to 100 eggs were exposed to 60 cc. of solution "for (not) more than twenty-four to thirty-six hours" following the eight-cell stage and then returned to sea water. Mention was not made of the methods employed, if any, to prevent evaporation.

In the present work tests were made to determine what precautions were necessary for the prevention of rapid evaporation. At 20° C. the partial pressure of ethyl alcohol in a 10% solution in water (by weight) is 28% of the total vapor pressure of the solution and at 40° C. is 34% (International Critical Tables). Initially comprising only 10% of the solution, the alcohol will contribute almost a third of the initial evaporation. The percentage of alcohol in the solution will fall rapidly if evaporation is allowed to take place. The salts in sea water will have no significant effect on the situation (personal communication from Professor G. B. Kistiakowsky). Stacking of finger bowls seemed inadequate to prevent evaporation of this magnitude and sealing the bowls would be time-consuming; a glass plate larger than the finger bowl was therefore laid over the aperture of each bowl. As a test of the effectiveness, covered and uncovered bowls were left 17 hours at 20.5° C. in a laboratory room. Each initially contained 55 cc. of a 10% (by volume) solution of absolute ethyl alcohol in sea water. At the end of the period the covered bowl contained 54.75 cc. (the difference from the initial volume being within the limits of error of measurement); the uncovered bowl contained only 48 cc. Since in the experiments solutions were replaced every 12 to 17 hours, and the dishes were covered, evaporation was not significant.

The experiments with alcohol were carried out in much the same way as those with $MgCl_2$. Unripe and unfertilized eggs were removed at the start of the experiment so that only eggs that were initially developing were tabulated. The embryos were returned to sea water in the 1949 series after 24½ to 33½ hours, and in the 1955 series after 51 to 53½ hours of exposure to the alcohol mixtures. In the

latter case the embryos were left in the alcohol solutions until they could be seen to be developing eyes in the less lethal concentrations. Experiments in 1949 were at temperatures most frequently between 23° and 25° C. as noted for the $MgCl_2$ experiments, and in 1955 entirely between 21.5° and 22.8° C. Lethality of the solutions is summarized in Table IV.

It should be noted that the two embryos counted as having survived the 5% alcohol treatment in reality failed to axiate. In these two cases the yolk spheres did not disintegrate and unorganized masses of cells with a few chromatophores developed upon their surfaces. The 5% alcohol mixture thus for all practical purposes was lethal. This held true when it was used for 32 hours or for 51½ hours (the two surviving eggs were in the latter group).

The 2% and 2½% solutions (1,268 embryos) produced only about a dozen perfect cyclopeans, which did not hatch. A dozen embryos in these bowls did hatch, including one synophthalmic embryo. The 3% solution proved best for production of cyclopia while still allowing moderately good development of body form. In the 1949 series with 3% alcohol, embryos were tabulated individually,

TABLE IV

1949 and 1955 alcohol experiments

No. of bowls	% of alcohol	Total embryos	% embryos dead before eyes developed
4	2%	650	5%
3	2½	618	4
9	3	692	29
4	4	697	73
3	4½	608	89
4	5	869	99.8
		4,134	

the living embryos giving the following totals: 128 cyclopeans including some with a single optic cup but two lenses, 43 monophthalmics, 60 two-eyed embryos including some synophthalmics which had two optic cups, each with lens and pupil, and 26 individuals that could not be classified. None of the embryos treated with 3% alcohol hatched. The 4% solution yielded an even higher percentage of cyclopean embryos among the survivors, but general body development was so inhibited and so many of the eyes were greatly reduced in size that results did not compare favorably with those at 3% concentration. The 4½% solution allowed only abortive attempts at axiation, and as noted above, the 5% solution was lethal.

DISCUSSION

The first point that is demonstrated by the present data is that the $MgCl_2$ method will not ordinarily give large percentages of cyclopean embryos. McClendon (1912) stated (p. 139), "In numerous experiments covering an entire season, I failed to obtain as high a per cent of cyclopia with magnesium chloride as recorded by Stockard." Stockard (1909) admitted difficulties in repeating his

initial experiment. Yet McClendon's work has largely been overlooked because of its negative character. Stockard classified all grades of synophthalmia as cyclopia, and yet the percentages of *all* abnormals in the best instances in the present experiments remain at about one-tenth of the fifty per cent cyclopia reported by Stockard. In Table III the numbers of cyclopean embryos probably are too high, since whenever there was doubt in cases where the eye seemed to be cyclopean but was so reduced as to make it difficult to be certain, the embryo was classified "cyclopean" rather than "other abnormal." The totals for *all* abnormals, however, are accurate.

The chances of obtaining free swimming cyclopean fish are not as good as the tabulated figures would make them appear to be. Only three cyclopean and four synophthalmic embryos hatched in the 1955 experiments. In addition, 21 monophthalmics, 17 with one eye partially reduced, and three anophthalmics hatched. All of these came from Series A except for three monophthalmics and two with one eye partially reduced from Series B, and one synophthalmic and two monophthalmics from untabulated bowls started after the temperature-controlled experiments and upon which individual records were not kept. No abnormals from the temperature-controlled experiments (Series C) hatched, a fact for which no explanation is apparent.

The influence of temperature in the $MgCl_2$ work now seems clear. Loeb (1915) and Kellicott (1916) reported some normally developing Fundulus embryos after eggs had been exposed in the early stages for a time to 7° C., and 8 to 10° C., respectively. Many embryos died or were markedly abnormal after such exposure, however. In the present work it is clear that the 19/60 $MgCl_2$ mixture couples its effect with that of cold in an additive way, for not one embryo survived of 1,429 exposed to 10° C. in the $MgCl_2$ solution (Series C, Table III). Most of them reached the late blastula stage before disintegrating. At temperatures for the most part of 17 to 18° C., between 5 and 6% of 3,395 surviving embryos in 19/60 $MgCl_2$ were abnormal (Series A and C). At temperatures for the most part of 20 to 22° C., 2.6% of 3,582 surviving embryos in 19/60 $MgCl_2$ were abnormal (Series B). Finally at temperatures between 25 and 26° C. only 0.9% of 1,166 surviving embryos in 19/60 $MgCl_2$ were abnormal (Series C). Unfortunately there were no means to test temperatures between 11 and 16° C. There remains the possibility that the optimum temperature for obtaining cyclopean embryos in the $MgCl_2$ solutions lies within this range.

In addition to the temperature differences there is the possibility that differences in hereditary susceptibility may cause variation in results. The incidence of any abnormality was so low in the 16 bowls kept at 25° C., for which there were control bowls kept at 18° C., that no significant results were obtained. Nevertheless, one cannot fail to be impressed with the great variation that does occur among batches of eggs handled in exactly the same way under the same conditions. The 19/60 $MgCl_2$ tests in Series A, Table III, are a good example. Of 58 cyclopean embryos, 46 occurred in three bowls, and of the total of 23 bowls, 14 contained no cyclopeans at all.

The results of the alcohol work indicate that Stockard did not control evaporation to any extent since he found 3% to 9% alcohol to be effective in producing living cyclopean embryos, and the present work shows 5% alcohol to be lethal if that concentration is maintained. Desired results may be obtained in percentages

of alcohol steadily decreasing with time, but conditions cannot be repeated without control of humidity, temperature, and air currents.

Summary

1. Under the conditions of the present $MgCl_2$ experiments, which followed those of Stockard as closely as possible except for temperature control, 2.0% of 11,912 surviving embryos were cyclopean; 0.3% were perfect cyclopean with an eye close to normal size, of which only three individuals hatched.

2. The optimum concentrations of $MgCl_2$ at the temperatures of these experiments were those which Stockard designated 18/60, 19/60, and 20/60 *M*.

3. With 19/60 *M* $MgCl_2$ solution, 10° C. proved lethal, while 17 to 18° C. was the optimum temperature used, with effectiveness falling as the temperature increased to 26° C. It was not possible to test temperatures of 11 to 16° C.

4. At temperatures of 21.5 to 25° C., 3% alcohol in sea water was the most effective in producing cyclopean embryos with moderately well developed body form; 4½% alcohol allowed only abortive axiation and 5% alcohol was lethal.

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MATING TYPES IN OXYTRICHA AND THE SIGNIFICANCE OF MATING TYPE SYSTEMS IN CILIATES[1]

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Sonneborn's (1937) discovery of mating types in *Paramecium aurelia* made possible a new approach to the classical and perplexing problems of sexuality, inheritance, and life cycles in ciliated protozoans. Mating types were later found in other species of this genus (Sonneborn, 1938; Jennings, 1938; Giese, 1939; Gilman, 1939) and in *Euplotes* (Kimball, 1939), *Stylonychia* (Downs, 1952), and *Tetrahymena* (Elliott and Gruchy, 1952; Nanney and Caughey, 1953). The present communication reports mating types in a fifth genus, *Oxytricha*. The mating behavior and some aspects of the life cycle of *O. bifaria* will be described. Finally, speculations as to the functions and significance of the known systems of binary and multiple mating types in ciliates will be presented.

Materials and Methods

Specimens of fresh water collected from natural sources were found to contain the hypotrich, *Oxytricha bifaria*. Four stock cultures, each representing the progeny of a single cell, formed the initial material for this investigation. A list of the stocks and their sources follows: stock 4 from Bloomington, Ind.; stock 14 from Ann Arbor, Mich.; stocks 24A and 24C from Locksley Station, Pa. These cultures can be induced to conjugate with one another and, together with their sexual progeny, furnish nine mating types to be described below. Additional collections provided new stocks belonging to one or another of these mating types.

In general, the methods described for handling and culturing *Paramecium aurelia* (Sonneborn, 1950) were successfully adopted for studies of *Oxytricha*. Mass cultures and isolation lines were routinely maintained in lettuce or Cerophyl infusions inoculated with *Aerobacter cloacae*. Under these conditions the present strains of *Oxytricha* have given, after three years of observation, but few indications of cyclic periods of depression or of senescence and death reported for other pedigrees (see Jennings, 1929). But it is important to note that some stocks and hybrid clones (obtained from crosses between the original stocks) lost their initial vigor, became senile and finally died. To induce conjugation, diverse cultures were provided sufficient nutrient to allow 0.5 to 1.0 fissions per day, and were mixed in a depression slide when they had exhausted most of the available bacteria. Mating began about two hours after the mixture was made; if well-fed cultures were mixed, the occurrence of conjugation was delayed until the food supply had been further exhausted. When one or both of the cultures were allowed longer periods of

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starvation before mixture, the cells usually became cannibalistic. Cannibals soon attained abnormally large sizes and could not be induced to conjugate. Under optimal conditions, 25 to 95 per cent of the cells formed pairs. Conjugation can apparently take place at any hour of the day and at temperatures ranging from 17 to 32 degrees C.

Nuclear reorganization in the present strains is apparently limited to conjugation; attempts to induce autogamy by limiting the food supply of mature clones proved unsuccessful.

Results and Discussion

In *Paramecium,* true conjugation can take place only between cells of complementary mating types. But in *Euplotes,* cells of a given mating type will conjugate when exposed to filtrates from cells of other mating types (Kimball, 1939) and, for this reason, mixtures of diverse cultures may come to contain induced "selfing" pairs together with other pairs consisting of cells of complementary mating types.

Table I

The breeding system in O. bifaria

(The plus signs signify that conjugation normally occurs in mixtures of the types indicated)

	I	II	III	IV	V	VI	VII	VIII	IX
I	−	+	+	+	+	+	+	+	+
II		−	+	+	+	+	+	+	+
III			−*	+	+	+	+	+	+
IV				−	+	+	+	+	+
V					−	+	+	+	+
VI						−	+	+	+
VII							−	+	+
VIII								−	+
IX									−

* Small numbers of selfing pairs frequently observed.

Pairs formed following the mixture of diverse cultures of *Oxytricha* regularly consist of cells of diverse types. This conclusion is based on the following facts: (1) Animals cultured in lettuce infusions for a few days appear darker than others grown in Cerophyl infusions. Appropriate mixtures of "dark" and "light" cells provided conjugations in which each pair consisted of a dark cell mating with a light cell. (2) Soon after they join, pairs may be forced apart by repeatedly expelling them from a micropipette. The mating types of members of such split pairs were always found to be complementary. (3) Attempts to induce "selfing" by exposing cells to filtrates from cells of other types have failed.

However, not all conjugations in *Oxytricha* involve cells of complementary mating types. Some cultures may self whenever conditions for mating are proper. For example, cultures of stock 24A, mating type III (see Table I) regularly contain about one per cent mating cells. The progeny of split pairs obtained from such type III cultures behave as the parental culture; each new culture will self, but is predominantly type III. Hence, it is possible that selfing pairs consist of

cells of the same type induced to mate by circumstances as yet unknown. Alternatively, selfing cultures may consist of a small number of cells of an unstable mating type complementary to the predominant and more stable type, a situation which would result in selfing.

The remarkable mating behavior of *Oxytricha* prior to conjugation merits description despite similarity to the "Paarungsspiel" previously described for *Stylonychia*, a closely related genus, by Grell (1951). When cultures of complementary types are mixed the activity of the cells remains unchanged for about two hours. Thereafter, small groups of cells collect on the bottom of the culture dish and within each group the cells describe contiguous circles. Soon two such "dancing" cells meet at their anterior adoral surfaces and become relatively quiescent. The mates so joined may presently separate and find other partners or become more firmly united. If conjugation proceeds each cell is supported on the bottom of the dish by its posterior cirri, and the pair forms an obtuse angle. This angle is slowly reduced so that the cells finally adhere tightly along most of their lengths and conjugation is completed in this position. Mating behavior in *Euplotes* has been found to conform in general to the description given above (Kimball, personal communication; Siegel, unpublished). The complicated pattern of mating behavior observed in these organisms suggests that such cells bear a "sticky" stuff on localized surface areas which must be brought together in order to initiate conjugation. In contrast, other ciliates such as *Paramecium* form large clumps of mating cells; these animals are apparently sticky all over, initial unions are non-specific, and mating behavior is comparatively simple and direct.

Nine mating types are presently known, although relatively few stocks and hybrids from but two crosses have been studied as yet (Table I). Each type will conjugate with all of the other eight types but, with the exception of type III, will not self in unmixed controls. It seems quite likely that new stocks and other hybrids will provide additional mating types. Whether the species *O. bifaria* encompasses genetically isolated subspecies or "varieties" at least superficially comparable to those in *Paramecium aurelia* and *P. bursaria* remains to be discovered.

Some crosses (I × II, III × V, V × VII) yield high proportions of viable hybrids while the majority of the hybrids of other crosses (I × III, II × V) die. Hybrid mortality could be due either to strain differences or to the incompatibility of certain mating types *per se*. In contrast to these results, selfing is almost invariably followed by death, even in the case of cultures which can be successfully outcrossed. This fact suggests that lethal factors, responsible for mortality in both selfing and certain outcrosses, may be discovered in further genetic analyses of breeding relations.

Oxytricha exemplifies the ciliate life cycle first described in Maupas' (1888; 1889) monumental papers and intensively studied by Jennings (1944) in *Paramecium bursaria*. Starting as exconjugants, clones pass through stages of immaturity, adolescence, maturity, senescence, and finally die. Following the separation of conjugants, the new clones undergo periods of vegetative reproduction during which they cannot be induced to mate and hence are called "immature." Adolescence is marked by the ability of the new clone to conjugate with some, but not all, of the established mating types. Moreover, when these cultures can be

induced to mate they yield relatively small numbers of conjugating cells. The stages of immaturity and adolescence may last for only a month or may persist for as long as two years. After further reproduction the clone acquires the capacity to mate readily with representatives of all the mating types, save those of its own type, and is now mature. A few hybrid cultures have passed from maturity to senescence and death, but most remain mature for at least two years. The persistence of each of these life cycle stages reflects a pattern of changing cell heredity or progressive differentiation under the control of genetic factors which are as yet unknown. In view of the various hypotheses for the general phenomenon of aging (Jennings, 1929; Sonneborn, 1954; 1955), it would be of interest to re-investigate this well-known problem, taking advantage of controlled conjugations between strains characterized by dissimilar life cycles.

The life cycles of certain ciliates may be greatly lengthened or perhaps eliminated entirely with the discovery of proper nutritive media and culture conditions. For example, clones of *Tetrahymena* do not normally pass through senescence and die off, although, parenthetically, they do undergo definite stages of sexual immaturity followed by adolescence and maturity. The following discussion pertains to other ciliates which show a more or less well-defined and complete life cycle. The important point for our purpose is that under conditions in which such a complete life cycle appears, fertilization has a rejuvenating effect. In these organisms senescence and death seem inevitable in the absence of nuclear reorganization, either uniparental autogamy (perhaps described as endomixis in some forms) or conjugation. If clones are aged, conjugation results in the death of most or all of the enconjugants (Jennings, 1944) and rejuvenescence occurs in only a few or none of the exautogamous progeny (Pierson, 1938; Sonneborn, 1954). Therefore, the periodic occurrence of some form of nuclear reorganization resulting in the formation of new clones would appear essential for the successful maintenance of such ciliate species. Moreover, since these processes may provide novel genotypes and genetic recombinations, they could be of some evolutionary consequence. The development of mating types must have been influenced by these fundamental factors. The significance of systems of binary mating types, as in *P. aurelia* and *P. caudatum*, and multiple mating types, as in *P. bursaria* and *Oxytricha*, will be considered in the next paragraphs.

All else being equal, species with a system of multiple mating types would be expected to conjugate more frequently than species with a system of binary mating types, for contacts between cells of complementary mating types would occur more often. The inadequacy of conjugation as the sole mechanism for nuclear reorganization in species with systems of binary mating types may have led to the development of a process for uniparental nuclear reorganization, namely, autogamy. Or the reverse may have occurred; species which failed to evolve autogamy developed compensatory systems of multiple mating types. At any rate, the two species of *Paramecium* for which binary mating types are known (*P. aurelia* and *P. caudatum*) pass through autogamy, whereas in those three species with multiple mating types (*P. bursaria, P. multimicronucleatum, P. trichum*) autogamy is extremely rare or lacking. Autogamy has not been reported to date in *Euplotes patella, Stylonychia putrina*, or *Oxytricha bifaria* despite intensive efforts, in some cases at least, to detect its occurrence. These species manifest multiple mating

types. The available data thus agree with the interpretation of a causal relationship between the kind of mating system and autogamy. Clearly many more species must be investigated before this hypothesis can be adequately evaluated. For example, it would be important in this connection to discover mating systems for *Stylonychia pustulata* and *Paramecium polycaryum*, species in which autogamy has been described by Fermor (1913) and Diller (1954), respectively. On the other hand, one of the six known varieties of *P. bursaria* is enigmatic, for only two mating types have as yet been collected from natural sources. Furthermore, endomixis has been reported for certain cultures of *Oxytricha bifaria* by Kay (1946); massive conjugation epidemics could be induced in wild cultures and mating was regularly preceded by the appearance of a few scattered animals described as endomictics. Unfortunately, this worker was unable to demonstrate mating types among these and other cultures.

Finally, it should be emphasized that although ciliates such as *Tetrahymena* do not pass through stages of senescence and death under artificial laboratory conditions, they may complete the life cycle in natural habitats. If so, the interfertile clones of *Tetrahymena pyriformis* studied by Nanney and Caughey (1953), which form a pattern of multiple mating types and do not pass through autogamy, would lend support to this hypothesis. The fact that *Tetrahymena rostrata* undergoes autogamy (Corliss, 1952) invites an investigation of the mating reactions in that species.

An entirely different explanation for the alternative systems of mating types should be considered. Multiple mating types may occur among species whose natural populations are small, thereby enhancing the chances for cells to encounter suitable mates. On this hypothesis species with binary mating types would be expected to be relatively more populous. However, adequate population studies have not yet been made and the significance of mating systems remains a matter of speculation.

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Summary

A study of *Oxytricha bifaria* revealed a system of nine interbreeding mating types. The mating behavior and life cycle of this organism are described. Two hypotheses which account for systems of multiple mating types in some ciliates and binary mating types in others are presented.

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